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SISTEMÁTICA Y FILOGEOGRAFÍA DEL CORAL DE PROFUNDIDAD *DESMOPHYLLUM DIANTHUS* (ANTHOZOA, HEXACORALLIA): INDICIOS MORFOLÓGICOS Y MOLECULARES

SYSTEMATICS AND PHYLOGEOGRAPHY OF THE DEEP–SEA CORAL *DESMOPHYLLUM DIANTHUS* (ANTHOZOA, HEXACORALLIA): MORPHOLOGICAL AND MOLECULAR EVIDENCES

Memoria presentada para optar al título de Doctora en Ciencias Biológicas con Mención Internacional por la Universidad Autónoma de Madrid por

Anna Maria Addamo

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Alla mia amata famiglia,

Odi et amo. Quare id faciam fortasse requires.

Nescio, sed fieri sentio et excrucior.

Catullo

Labor improbus omnia vincit.

Virgilio

Audaces fortuna iuvat.

Virgilio

Odio e amo. Forse mi chiederai come sia possibile;
non so, ma è proprio così, e mi tormento.

Odio y amo. Quizás te preguntes por qué hago esto.
No lo sé, pero siento que así ocurre y me torturo.

I hate and I love. Perhaps you ask why I do this?
I do not know, but I feel it happen and I am torn apart.
Catullo

Il lavoro duro vince ogni cosa.

Un trabajo ímprobo todo lo vence.

Steady work overcame all things.
Virgilio

La fortuna aiuta gli audaci.

La fortuna favorece a los valientes.

Nothing ventured, nothing gained.
Virgilio

Gratiarum actio

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RESUMEN

El conocimiento científico de la filogenia, filogeografía y genética de poblaciones de los corales del orden Scleractinia, se basa principalmente en estudios llevados a cabo en especies de aguas someras y tropicales. Sólo unos pocos estudios incluyen corales sin zooxantelas simbiontes y de aguas profundas, si bien sus especies constituyen la mitad del número total de las especies de corales identificadas hasta el momento.

Desmophyllum dianthus (Esper, 1974) es un coral solitario, azooxantelado y de profundidad, perteneciente a la familia Caryophylliidae. La especie ha sido objeto de cierta atención en estudios recientes, aunque los aspectos sobre sus relaciones filogenéticas y la estructura genética de sus poblaciones han sido explorados marginalmente . Por tanto, el objetivo central de esta Tesis es precisamente analizar la relación filogenética de la especie a nivel inter- y intrafamiliar, y la genética de sus poblaciones, todo ello a través de un enfoque multidisciplinario.

Con el fin de alcanzar este objetivo, teniendo en cuenta que el género pertenece a la familia Caryophylliidae, bien conocida por su carácter polifilético, se han analizado distintas especies de cariofílidos con marcadores moleculares comúnmente utilizados en los estudios de relaciones filogenéticas del orden Scleractinia y, además, se han desarrollado nuevos marcadores gracias a la aplicación de metodologías de secuenciación masiva.

A nivel intraespecífico, puesto que *D. dianthus* es uno de los escasos corales ampliamente distribuidos, se han podido analizar individuos de distintas áreas de ambos hemisferios, norte y sur. Para este estudio de diferenciación genética poblacional a escala global, se han empleado 30 nuevos microsatélites desarrollados a través de técnicas de pirosecuenciación. Por otra parte, y con el fin de delimitar el alto nivel de variabilidad morfológica que caracteriza a *D. dianthus*, se han llevado a cabo análisis de caracteres morfológicos de los esqueletos y de los pólipos.

Finalmente, se ha secuenciado el genoma mitocondrial completo de un ejemplares del mar Mediterráneo y otro del suroeste del Pacífico, realizando análisis comparativos con especies próximas, con el fin de aclarar la relación filogenética de ciertas especies estrechamente relacionadas.

Los resultados revelaron que *D. dianthus* pertenece filogenéticamente al grupo 'robusto' de los escleractinios, en uno de los clados polifiléticos de Caryophyllidae. Por su parte, su extremadamente alta variabilidad morfológica no ha mostrado patrones ecológicos o geográficos. En cuanto a la estructura genética de sus poblaciones, se ha detectado una cierta diferenciación entre los ejemplares procedentes de los hemisferios norte y sur, con un modelo de flujo génico de aislamiento por distancia. Las corrientes profundas parecen desempeñar un papel clave en la dispersión de las larvas, creando peculiares barreras o, por el contrario, conectividad genética entre las poblaciones, como ocurre con las de Nueva Zelanda y Chile, que presentaron características genéticas propias, o las de Australia y Argentina, entre las que se ha detectado un cierto flujo génico, a pesar de la gran distancia geográfica que existe entre las dos regiones. Por último, se ha encontrado una sorprendente similitud genética, a través de diferentes marcadores moleculares de origen nuclear y mitocondrial caracterizados por diferentes tasas de mutación y niveles de polimorfismo, entre *D. dianthus* y el principal coral constructor de arrecifes de mares profundos *Lophelia pertusa.*

La comunidad científica todavía tiene que enfrentarse a muchas cuestiones pendientes sobre la filogenia y filogeografía del orden Scleractinia y, sobre todo, se requieren muchos más datos y análisis adicionales. Los resultados de este estudio ofrecen la primera información detallada sobre la filogenia y la genética de poblaciones de *Desmophyllum dianthus*. Además, la similitud genética encontrada entre esta especie y *Lophelia pertusa*, incide en la necesidad de una revisión taxonómica profunda de la familia Caryophylliidae y de sus géneros. En esta Tesis se han desarrollado nuevas herramientas moleculares, que han permitido conocer de forma más precisa los procesos que han modulado la historia evolutiva de *D. dianthus*, y que pueden resultar igualmente útiles para el análisis de especies cercanas.

Palabras claves: Scleractinia, coral de profundidad, *Desmophyllum dianthus*, filogenia, genética de poblaciones, marcadores moleculares, genómica, secuenciación masiva, morfología.

SUMMARY

Scientific knowledge on phylogeny, phylogeography and genetic population of scleractinian corals is mainly based on studies carried out on shallow water and tropical species. Only a few works involved deep-sea and azooxanthellate corals, whose species composed half of the total number of corals identified so far.

Desmophyllum dianthus (Esper, 1794), is a solitary azooxanthallate deep-sea coral, classified in the family Caryophylliidae. Recently, the species has received some attention in biological studies, although its phylogeny and population genetics has only been slightly explored. The aim of this Thesis is to analyse its phylogenetics relationships at inter- and intra-familiar levels, and its population structure, through a multidisciplinary approach.

In order to achieve the phylogenetic objective and because the genus belongs to the well known polyphyletic Caryophylliidae family, distinct caryophylliids species were analysed with molecular markers commonly used to study phylogenetic relationships of Scleractinia Additionally new ones were developed by high-throughput Illumina sequencing.

At intraspecific level, since *D. dianthus* is one of the few corals widely distributed, several individuals from different areas of both northern and southern hemispheres were analysed, with 30 new microsatellites developed by 454 pyrosequencing, giving a picture of its population genetics on a global scale. Additional analyses were performed with morphological characters of skeleton and polyps to define the high level of morphological variability that characterized *D. dianthus*. Furthermore, the complete mitochondrial genome of one specimens from the Mediterranean Sea and other from the southwestern Pacific were also sequenced. Comparative analyses are performed in order to clarify phylogenetic relationship of closely related species.

Results revealed that *D. dianthus* belongs phylogenetically to the scleractinian “robust group” in one of the Caryophylliidae polyphyletic clades, and its extremely high morphological variability has not showed either ecological or geographical patterns. Specific population structures are detected for northern and southern hemispheres, with an isolation by distance model of gene flow (IBD). Moreover, deep-water currents play

a key role on larval dispersal, creating peculiar genetic barriers or genetic connectivity between *D. dianthus* populations, such as New Zealand and Chile, whose populations presented their own genetic characteristics, or Australia and Argentina, whose gene flow is detected despite the large geographic distance between them. Finally, a surprising genetic similarity, throughout several nuclear and mitochondrial molecular markers characterized by different mutation rates and polymorphism level, was found between *Desmophyllum dianthus* and the main deep-water coral reefs builder *Lophelia pertusa*.

Even though the scientific community still have to face several unanswered questions over phylogeny and phylogeography of Scleractinia, and overall much more additional data and analyses are needed, the results of this study provide the first detailed insight on phylogeny and genetic population of *Desmophyllum dianthus*. Moreover, genetic similarity between *D. dianthus* and *L. pertusa* demonstrated the need for a complete taxonomic revision of the genera within the family Caryophylliidae. In this Thesis new molecular tools are provided to tackle this goal, both at the phylogenetic and phylogeographic levels.

Keywords: Scleractinia, deep-sea coral, *Desmophyllum dianthus*, phylogeny, population genetics, molecular markers, genomics, next generation sequencing, morphology.

INTRODUCTION

In this Thesis the different processes that could have influenced the evolutionary history of the stony scleractinian coral *Desmophyllum dianthus* at morphological and molecular level are going to be investigated. The applied methodology not only will combine traditional molecular and morphological techniques, but thanks to the progress in new and sophisticated technologies and methodologies - such as next generation sequencing and three dimensional coordinates of skeletal landmarks - new morphological and molecular characters will be searched.

Revolutionary Systematics of Scleractinia

The order Scleractinia includes the main coral reef-builder species. Scleractinian corals can be found throughout the world's oceans, including temperate and polar regions, and from intertidal to the deepest trenches. There are approximately 1,500 described extant species (Roberts *et al.* 2009), whose half are reef-building corals, largely colonial, and zooxanthellate and occurring in the clear, shallow waters of the tropics. The other half of the order comprises largely solitary and azooxanthellate species, occurring in all regions of the oceans, including the greatest depths.

'Though scientific interest in scleractinian corals originated in the 16th century, the knowledge base continues to grow and is far from complete' (Zlatarski and Stake 2012). This unsatisfactory feeling is reflected along the three periods in which history of scleractinian studies were described: plant period (1576-1727), animal period (1727-2007), holistic period (2007-present) (Zlatarski and Stake 2012). Their classification has been marked by confusion from the beginning, when corals were interpreted as plants by M. Lobel in 1570s and just 200 hundred years later were recognized as animal in 10th edition of Linnaeus's "Sistema Naturae" by Peysonnel in 1750s (Vaughan and Wells 1943). The fundamental base of classification of Scleractinia was formed by progressive evolution of techniques, characters, and specialists. Initially, palaeontologists led the 'Skeletal Phenetic Systematics' of Scleractinia, in which skeletal morphology and microstructure characters were applied to classify and describe the high variability of corals. Subsequently, thanks to the advent of SCUBA techniques, which made the underwater habitats accessible to researchers and allowed the their observation all around the world, the studies were extended in many others scientific disciplines - such as ecology, life history, and molecular biology - and biologists started to play a key role

in coral classification. While confusion about their classification diminished as corals were studied in greater detail at morphological level, such confusion re-emerged in the late 20th century when molecular techniques began to be applied to scleractinian systematics (Budd *et al.* 2010). In fact, both mitochondrial and nuclear DNA markers revealed inconsistencies with the conventional gross-morphology-based taxonomy, suggesting that the order is represented by two major lineages ('robust' and 'complex') and did not support the monophyly of traditional suborders and most traditional families (Chen *et al.* 1995; Romano and Cairns 2000; Zlatarski and Stake 2012). Thus, molecular phylogenetic analyses have revolutionized scientific understanding of scleractinian evolution (Budd *et al.* 2010), but the use of integrative taxonomy, in which molecular analyses were applied in combination with more sophisticated morphological studies, demonstrated to improve the scleractinian relationships resolution at different phylogenetic levels (Budd and Stolarski 2009; Benzoni *et al.* 2011; Arrigoni *et al.* 2012; Benzoni *et al.* 2012; Budd *et al.* 2012; Kongjandtre *et al.* 2012; Kitano *et al.* 2013; Arrigoni *et al.* 2014a; Arrigoni *et al.* 2014b; Arrigoni *et al.* 2014c; Benzoni *et al.* 2014; Kitano *et al.* 2014). The renewed interest in the micromorphological and microstructure characters suggested the possibility to harmonize skeletal and molecular data and, consequently, the importance of using a holistic approach (Stolarski and Roniewicz 2001) seems to be the key to obtain a classification of Scleractinia that closely reflects the real corals phylogenetic relationships. Recently, many studies revealed a large rearrangement of scleractinian families and genera; and new species are still being discovered, improving the new classification and paving the way for new hypotheses as well (Huang *et al.* 2011; Arrigoni *et al.* 2012; Benzoni *et al.* 2012; Budd *et al.* 2012; Benzoni *et al.* 2014; Huang *et al.* 2014a; Huang *et al.* 2014b; Kitahara *et al.* 2014; Kitano *et al.* 2014; Schmidt-Roach *et al.* 2014).

It is important to notice that molecular phylogenetic hypotheses, which differ significantly from traditional classification, have been primarily focused on reef-building corals; however, once a substantial number of azooxanthellate and deep sea corals were included in molecular phylogenetic analyses, the basal relationships within the order changed and shallow-water scleractinian corals appeared to have had several independent origins from solitary, azooxanthellate precursors (Stolarski *et al.* 2011). Other hypothesis (called 'naked corals hypothesis') on Scleractinia and

Corallimorpharia orders paraphyly, which could be not excluded yet, was suggested from nuclear and mitogenomic phylogenetics, where whole mitochondrial genomes showed an important difference between nucleotide-based and amino acid-based phylogenetic resolution (Medina *et al.* 2006; Kitahara *et al.* 2014; Lin *et al.* 2014).

In relation to the characters used for taxonomic classification and phylogenetic analyses, many of the most important morphological features traditionally used are related to the architecture of the corallite or corallum (basic morphological unit), but they also involved the morphogenesis of the skeleton, including the budding and integration of corallites within colonial corals (Wells 1956). Skeletal morphological features can be broadly grouped into three categories: 1) macromorphology: the size and shape of features related to corallite architecture and the integration of corallites within colonies, 2) micromorphology: the shape of teeth and granules along the margin and faces of septa; 3) microstructure: the arrangements of centers and fibers within septa and the corallite wall. While macromorphological characters are important in traditional taxonomy at the generic and specific levels, micromorphological characters are also important at the familial level and above (Budd and Stolarski 2009; Budd *et al.* 2010). There is another issue worthy of mention too, namely cnidome (the complement of cnida categories occurring in an anthozoan taxa). Although, it is still almost unexplored character in Scleractinia, recently studies demonstrated its potential utility in taxonomy and phylogeny as well (Fautin 2009; Martínez-Baraldés *et al.* 2014).

On the other hand, many of the most molecular data used in phylogenetic studies come from both nuclear and mitochondrial origin. Although ribosomal DNA (rDNA), including 28S, 5.8S, 18S and two inter-transcribed spacer (ITS1 and ITS2), is frequently used for molecular phylogenetic analyses at the species levels, there were several problems in applying rDNA markers to study *Acropora* (Chen *et al.* 1995; Chen *et al.* 2004), for instance. The importance encompassed in this genus is based on its dominance in coral reefs and worldwide distribution (Veron 2000). In addition to be the main member of spawning events, which characteristics suggest its unusual evolutionary process called ‘reticulate evolution’ (van Oppen *et al.* 2001), the genus *Acropora* has particular characteristics compared with all other corals, such as the highest number of species, extremely high evolutionary rates and high intra-individual

genetic variation, which prevents correct inference of the evolutionary processes (Vollmer and Palumbi 2004; Wei *et al.* 2006; Fukami 2008). Thus, mitochondrial genetic markers, as the mitochondrial rDNAs (16S and 12S), cytochrome oxidase subunit I (COI), cytochrome b (cytb), and ATPase6 (ATP6) were applied to many coral species (Stolarski and Roniewicz 2001; Fukami *et al.* 2008; Kitahara *et al.* 2010). However, analyses of the mitochondrial genome revealed that the evolutionary rates of its coding genes are 10 times slower in corals than in vertebrates (Shearer *et al.* 2002; Hellberg 2006), making difficult species-level analyses due to the low number of substitutions among species. Hence, mitochondrial non-coding regions were considered as potential candidates for species level, whereas the coding regions are used for higher taxonomic levels due to their slow rates. Thanks to the advance in genomics techniques, mitogenomics could be applied to a large number of taxa and has been demonstrated to have potential resolution at phylogenetic level (Curole and Kocher 1999; Weisrock 2012). In fact, mitogenomic approach has been recently applied to Scleractinia, providing a more complex perspective on interrelationships of scleractinian families and genera, showing that not only the substitutions, but also some rearrangements can provide valuable data (Kitahara *et al.* 2014; Lin *et al.* 2014).

Nevertheless, the use of other nuclear molecular markers that could confirm the systematics of Scleractinia is advisable to corroborate the phylogenetic reconstructions inferred so far. Although nuclear single copy gene markers (mini-collagen, PaxC, Calmodulin, Galaxin, and Histone 3) and nuclear multicopy gene markers (as β-tubulin gene) - including intron regions, which have much faster evolutionary rates - are supposedly more phylogenetically informative, these genes were not frequently used for phylogenetic analyses in corals (Flot *et al.* 2008; Fukami 2008; Fukami *et al.* 2008; Wirshing and Baker 2008; Huang *et al.* 2011; Arrigoni *et al.* 2012; Arrigoni *et al.* 2014b; Huang *et al.* 2014a).

Phylogeography and population genetics

Population genetics is the study of genetic variation within populations.. It involves the examination and modelling of changes in the frequencies of genes and alleles in populations over space and time, as the population is subjected to the four main evolutionary processes: natural selection, genetic drift, mutation and gene flow. When

the scientific knowledge about species is limited, or totally absent, studies on phylogeography and population genetics of the species allow inferring on biology of the species, such as reproduction strategy, asexual reproduction rate, larval dispersal, connectivity, and inbreeding, whose studies were not possible to conduct directly. Therefore, such studies increase the knowledge of intraspecific variation of species and its geographic distribution, allowing better definition of the species and more efficient conservations strategies management. Molecular data also contribute to understanding reef connectivity by demonstrating historic and current patterns of gene flow between populations (Hellberg 2007).

Early studies were conducted to test both nuclear and mitochondrial markers failed at species-level but resulted promising at individual level, such as non-coding regions. On one hand, nuclear markers revealed not to be very informative in terms of clustering individuals - except for the nuclear ITS2, which provided a slightly clearer picture than other nuclear gene introns, but apparently do not resolve all potential relationships (Chen *et al.* 2004; Flot and Tillier 2006; Flot *et al.* 2008). On the other hand, due to the unusual nature of coral mitochondrial genome in containing several non-coding regions respect to other metazoans, mitochondrial introns were considered potential candidates for population level analyses (van Oppen *et al.* 2002; Flot *et al.* 2008). Studies showed that putative control and open reading frame (ORF) were useful for resolving interpopulational relationships among Scleractinia (Flot *et al.* 2008), but the lack of full supported relationships lead to considered them not highly useful. From a population genetics point of view, nuclear microsatellite sequences are demonstrated to be the most revealing DNA markers available for inferring population genetic structure and dynamics (Estoup and Angers 1998; Estoup *et al.* 2002; Zhang and Hewitt 2003). Although microsatellite became the most used markers, the population genetics in scleractinian corals is still marginally explored in shallow water as well as in deep-sea corals (Le Goff-Vitry *et al.* 2004; Nakajima *et al.* 2010; Casado-Amezúa *et al.* 2011; Costantini *et al.* 2011; Morrison *et al.* 2011; Casado-Amezúa *et al.* 2014).

The stormy species concept in corals

The earliest species concept applied to corals was clearly typological (i.e. a species is a group of organisms conforming to a common morphological plan), emphasizing the

species as an essentially static, non-variable assemblage. Individuals do not stand in any special relation to each other, being merely expressions of the same type. From this point of view, variation is the result of imperfect manifestations of the idea implicit in each species (Mayr 1969; Lincoln *et al.* 1982). Species boundaries within the Scleractinia have historically been defined using discontinuities in morphological features of skeletal architecture. However, the modular nature of corals gave rise to two major sources of variation in skeletal structures, which have created long-standing problems for recognizing species within the group: extensive variation in corallite characters/dimensions within a single colony and in colony morphology among corals within a single described species (Willis 1990). Since extensive difference occurred within a seemingly uniform biotope and between different biotopes (Veron 1982), studies of habitats and environmental effects on skeleton characters, led to a broadening of species boundaries as 'ecomorph' (i.e. a intraspecific skeletal variation phenotypically and/or genotypically determined in response to specific ecological conditions (Veron and Pichon 1976)) adhered to the phenetic species concept (i.e. 'morphological differences that were considered by taxonomist describing the new pecies to be of sufficient magnitude to warrant specific status', Sokal 1973; Willis 1990). However, studies have clearly demonstrated that large variation in both corallite and corallum characters may be encompassed by a single species, either through phenotypic plasticity in response to a number of environmental factors, or through genetic polymorphism (Willis 1990). Therefore, it has been proposed that different forms of morphologically variable corals represent separate species based on evidence of divergence in their fertilization systems (Paterson 1988) suggesting reproductive isolation. Although the recognition species concept (i.e. a species is the most inclusive population of individual biparental organisms which share a common fertilization system, Templeton 1981) and biological species concept (i.e. species are groups of actually or potentially interbreeding natural populations, which are reproductively isolated from other such groups, Mayr 1942) started to take place, lack of reproductive data for many corals and the inappropriateness of breeding criteria precluded its application to the rich fossil record (Willis 1990), whose species - stable over geological time periods - have been defined according to evolutionary concept (i.e. 'a species is a single lineage of ancestral descendant populations, which maintains its identity from

other such lineages and had its own evolutionary tendencies and historical fate', Simpson 1961; Wiley 1978)). Although the evolutionary concept assumed that species are monophyletic in origin, it was considered preferable to adopt a broader species concept such as the phylogenetic concept (i.e. 'similarly to evolutionary concept it requires species to be monophyletic in origin, but incorporates pluralistic criteria for defining species limits, thereby accommodating asexual lineages', Mishler and Donoghue 1982), which was herein considered an appropriate species concept for scleractinian corals.

Desmophyllum dianthus: the elected scleractinian species

Desmophyllum dianthus (Esper, 1794), and its frequently used synomym *Desmophyllum cristagalli* (Milne Edwards & Haime, 1848), is the type species of genus *Desmophyllum* Ehrenberg, 1834, within the family Caryophylliidae Dana, 1846. Although at least 25 species have been described in this genus, most of them are currently considered as synonymies of the cosmopolitan *D. dianthus*, and only two are recognized as valid species: *D. striatum* Cairns, 1979, known from the western Atlantic at 130-823 m depth, and *D. quinarium* Tenison-Woods, 1879, known from the western Pacific at 36 m depth (Cairns 1995; Roberts *et al.* 2009).

D. dianthus is known to be an extremely variable species, with fewer diagnostic characters (e.g. no columella, no pali, or budding). It varies greatly in its corallum shape and diameter of attachment, ranging from serpentine to ceratoid and trochoid, often greatly flared, depending on its environment. Individual corallum larger than typical form, often produce pseudocolonial clumps of specimens, as the framework coral for deepwater coral bank (Cairns 1982). *Desmophyllum dianthus* is one of the few cosmopolitan species of Scleractinia, widely distributed across the Atlantic, Pacific and Indian oceans, but it is not present off Antarctica and the northern boreal Pacific. This solitary and azooxanthellate species occurs mainly on hard substrates from continental slope to the upper bathyal zone, commonly associated to scleractinian reef framework-forming cold-water corals *Lophelia pertusa* and *Madrepora oculata* (Roberts *et al.* 2009). Nevertheless, it has been also found attached to 'artificial substrates' as disused fishing nets, buoys and underwater cables and pipelines (Schembri *et al.* 2007). Its worldwide depth range is 80-2,460 m, exceptions made when environmental conditions

are favourable, namely cold upwelled waters associated with fjords (Roberts *et al.* 2009), as it occurs in New Zealand, where shallowest records are reported at 25 m (Cairns 1995), and in Chile, where single individuals are found as shallow as 8 m but larger accumulation are generally founded at depths around 20 m and deeper (Försterra and Häussermann 2003).

Except for taxonomic studies, earliest scientific works related to *D. dianthus* are dated to the late 1970s and were mainly focused on geological analyses, such as skeletal microstructure and ontogenetic development, lifespans and growth pattern and its potential paleo-climate proxies (Sorauf and Jell 1977; Stolarski 1995; Risk *et al.* 2002; Adkins *et al.* 2003; Montagna *et al.* 2006). Only in the last decade the scientific attention has moved to other disciplines and studies have been conducted to obtain insights in the biology, physiology and ecology of *D. dianthus* (Försterra and Häussermann 2008; Naumann *et al.* 2011; Thresher *et al.* 2011; Anagnostou *et al.* 2012; Maier *et al.* 2012). These studies have revealed that *D. dianthus* displays an average growth rate of 0.5-1 mm/yr with a long lifespan of up to 200 years (Risk *et al.* 2002), and resulting in being an exceptional proxy for pH, water mass temperature of marine ecosystem and relative history (Montagna *et al.* 2005; Montagna *et al.* 2006; Montagna *et al.* 2011; Anagnostou *et al.* 2012; Fillinger and Richter 2013). It seems to be a species with moderate thermal tolerance, and survival and growth of the specimens has been documented from 12ºC (general temperature for their development) up to 17ºC in the Mediterranean Sea (Naumann *et al.* 2014). Further, trophic ecology studies through aquaria experiments have highlighted zooplankton as the essential nutritional source for *D. dianthus*, important for supplying respiratory metabolism, skeletal growth and organic matter release, with further implications for the role of cold water corals in reef ecosystem functioning (Naumann *et al.* 2011). Recently, a one year-long term aquaria experiment study has estimated that *D. dianthus* displays significantly lower growth rates when maintained under acidified conditions, showing a lower tolerance to ocean acidification than other cold water corals species investigated up to date (Movilla *et al.* 2013; Movilla *et al.* 2014). Furthermore, investigations conducted in Chilean fjords along vertical (water depth) and horizontal (fjord mouth to head) transects including pH, salinity, oxygen concentration and temperature, have revealed a gradient in both (vertical and horizontal) orientation, and the capability of *D. dianthus* to grow under

aragonite saturation states close to one (even though with low calcification rates) and a pH range 7.7-8.2 detected along the fjord (no correlation was shown with calcification rates). Coping with such variations in water chemistry variations may become even more important, according to the predicted climatically induced changes in carbonates water chemistry (Addamo *et al.* 2012; Fillinger and Richter 2013; Jantzen *et al.* 2013).

The worldwide distribution of *D. dianthus*, studies about its demography, population structure, and reproduction are pretty much non-existent. Only recently a genetic study, using the internal transcribed spacers (ITS), the 16S mitochondrial ribosomal subunit (16S) and the control region (MtC), was conducted to determine levels of gene flow within and among populations of *D. dianthus* in Southern Pacific Ocean, as well as to assess the ability of corals to disperse into different regions and habitats (Miller *et al.* 2011). Results showed significant genetic subdivision among Australia, New Zealand and Chile, three widely separated geographic regions, consistent with isolation and limited contemporary gene flow. Strong differentiations were also showed among corals from different depth strata even on the same or nearby seamounts, indicting limited vertical larval dispersal. Therefore, it was hypothesized that the reproductive traits of *D. dianthus* might be similar to other deep-water corals, such as broadcast spawner with lecitotrophic larvae and thus low distance dispersal (Waller *et al.* 2005; Miller *et al.* 2011).

As above-mentioned*, D dianthus* is commonly associated to the deep-sea reef forming corals *Lophelia pertusa* and *Madrepora oculata,* and one important aspect that should be also considered is the human impact on deep-sea coral reef communities. Anthropogenic activities in the deep sea are not limited to bottom trawling by fishing activity; deep-seabed mining is now becoming a reality and with improved submarine techniques and international demand for metals at an all-time high, it seems likely to expand by exploiting mineral deposits over and within deep sea floor. Overlying all these activities, the effects of climate change may dramatically alter the marine environment (Roberts *et al.* 2009).

Given these considerations, the lack of a general and consensus definition of species concept in Scleractinia, as well as the presence of an extremely high morphological variability of *D. dianthus*, led to highly consider the importance of improving the

scientific knowledge on systematics and population genetics of *D. dianthus.* Genetic studies concerning differentiation of the species at interspecific and intraspecific level would serve as inference of diversity, population structure and reproductive traits, suggesting conservation management measures to prevent and/or reduce the relentless negative effects of human impact on deep-sea coral communities. Moreover, shaping such genetic diversity with the morphological variation could give and idea of the relevance of these features in the evolutionary history of the central target of this Thesis, *D. dianthus*.

HYPOTHESES & OBJECTIVES

The overall hypotheses set out to investigate in this Thesis and objectives planned for this purpose are the following:

Hypothesis 1: *Desmophyllum dianthus* and *Caryophyllia smithii* (Caryophylliidae), were described as a sister clade to *Stenocyathus* (Guyniidae), forming one of the polyphyletic groups of Caryophylliidae among 'robust corals' (Kerr 2005), although data from putative close species are missing.

Objective: To estimate *D. dianthus* phylogenetic relationships, four molecular markers among nuclear (large ribosomal RNA subunit 28S, and the internal transcribed spacer regions ITS) and mitochondrial (large ribosomal RNA subunit, 16S, and cytochrome c oxidase subunit I, COI) will be used including other representatives of caryophylliids. (Chapter I).

Hypothesis 2: Since no much data is available on phylogeography of deep-sea corals, it is hypothesized that *D. dianthus* could present similar genetic structure to that estimated for *Lophelia pertusa*, which should not be considered as one panmictic population but instead forms distinct populations (offshore and fjord) (Le Goff-Vitry *et al.* 2004).

Objective: In order to assess phylogeographic structure of *D. dianthus*, populations from northern and southern hemisphere will be tested with two molecular markers (mitochondrial 16S and nuclear ITS). (Chapter I).

Hypothesis 3: High morphological variability of *D. dianthus* was defined as dependent from environmental condition, where type of substrate and water turbidity can lead the corallite morphology from cylindrical to trochoid (Zibrowius 1980).

Objective: To confirm environmental-dependent morphotypes, morphological characters of several specimens of *D. dianthus* will be analysed combining macromorphological descriptive and 3D-geometric morphometric characters of skeleton, as well as diversity and distribution of organic character of polyps (cnidocysts). (Chapter II).

Hypothesis 4: Population genetics of species may be well detected by using molecular markers with high levels of polymorphism and mutation rate, such as short tandem repeats (STRs) or microsatellites (Estoup and Angers 1998).

Objective: To test the variability and utility of STRs in *D. dianthus*, microsatellites will be developed (Chapter III), and used to define the genetic variability, polymorphism and structure of *D. dianthus* populations from northern and southern hemispheres. (Chapter IV).

Hypothesis 5: Molecular markers commonly used in Scleractinia systematics may be not variable or informative enough for detecting genetic divergence among very closely related coral species (Fukami 2008).

Objective: To evaluate the utility of the markers, a review of phylogenetic signal will be reported for all molecular markers usually employed in phylogenetic studies of Scleractinia. Moreover, new molecular markers will be also developed and characterized for different phylogenetic levels in scleractinian evolution. (Chapter VI).

Hypothesis 6: Due to the high genetic similarity detected in previous analyses (Addamo *et al.* 2012), *D. dianthus* and *L. pertusa* are not two clearly distinct genera and in-deep revision should be necessary.

Objective: To explore the genetic similarity between *Desmophyllum* and *Lophelia*, complete mitochondrial genome from two specimens of *D. dianthus* will be sequenced and comparative analyses between both genera will be performed including other molecular markers previously developed, such as microsatellites and protein-coding genes. (Chapter VI).

MATERIAL & METHODS

Although detailed information will be presented in each chapter, a brief review of the study area, material and methods applied in this PhD Thesis is following reported:

Study Area

The study area analysed in this thesis included the deep-sea coral reefs located in the Mediterranean Sea, and the Atlantic and Pacific Oceans.

Cold-water coral reefs and coral carbonate mounds are morphologically formed through complex interactions between biological and geological processes under suitable hydrodynamic conditions (Roberts *et al.* 2009). Major frameworks constituents of the limestone structure, or also termed bioherms, are the stony corals. Even though deep-water coral reefs share many biological and physical features with tropical shallow water reefs, there are significant differences: deep-water reef-forming corals do not contain zooxanthellae and live in total darkness and in cold water, by preying on zooplankton that drifts through currents (Rogers 2004; Hovland 2008). Furthermore, deep-water corals have special environmental requirements that determine their distribution and growth form at all scale: hard surface associated with permanent or episodically strong currents, which rely on a vigorous flow of water to supply them with food, disperse eggs, sperm and larvae, remove waste products and keep the surface of corals free of sediments. Deep-water coral reefs occur mainly on the continental slope within a range 100-2,000 m depth, but they can also be found in seamounts, plateaus, ridges and the submerged side of oceanic islands. Exceptions to the rule were reported in fjords systems where they can be found at 20-40 m depth (Rogers 2004; Hovland 2008). From a biological point of view, the main reef-building organism is the stony coral *Lophelia pertusa*, often associated with other reef-builders, such as *Madrepora oculata* and *D. dianthus*, creating large coral reef provinces as a "unique biodiversity hot-spots" at mid-ocean depth (Rogers 2004; Hovland 2008).

Material and sampling methods

All corals analysed in this Thesis were sampled during oceanographic cruises conducted from 2006-2013 at several localities in the Mediterranean Sea, and the Atlantic and Pacific Oceans. Samples designated for molecular analyses were directly preserved in absolute ethanol; instead, those chosen for morphological studies of soft parts were

initially conserved in formaldehyde 4% and subsequently in absolute ethanol. Additionally, coral and tissue samples were also loaned by museum collections, such as the Smithsonian National Museum of Natural History (NMNH), Washington DC, the Muséum National d'Histoire Naturelle (MNHN), Paris, and the Instituto Español de Oceanografía (IEO), Spain.

A range of deep-water catching approaches does exist, but not all of these can sample cold-water corals habitats effectively; especially those scleractinian reefs where substrata contains hard coral frameworks and may even be lithified (Roberts *et al.* 2009). The main sampling methods applied to collected specimens then analysed in this Thesis are the following:

- Dredge: epibenthic dredge, epibenthic dredge Antolini, gear sled (Figure 1b).

- Trawl: epibenthic trawl, trawl-large beam, otter trawl Marietta (Figure 1c-d).

Dredges and trawls were the mainstay of deep-sea sampling during the nineteenth and most of the twentieth centuries, but there is now a consensus that they should be avoided in cold-water coral habitats. These devices not only capture groups of organisms from a range of habitats and facies altogether, but they are also very destructive (Roberts *et al.* 2009).

- Box corer and grab (Figure 1a-e).

Box corers were designed to sample the sediment-water interface and are often inappropriate in cold-water corals habitats, where coral fragments or glacial drop stones prevent cores from penetrating (Roberts *et al.* 2009).

As Roberts *et al.* (2009) indicated, recent development in video-directed, hydraulically controlled grab sampling may allow coral colonies and reef framework to be sampled and stored in a sealed grab so that the attached fauna is not lost while the sample is brought back through the water column.

- Remote Operated Vehicle (ROV) (Figure 1f).

ROV can sample specific habitats by either collecting selected organisms with manipulator arms or suction tube, thus it could be considered the most efficient sampling technique that allows the coral reefs damages to be minimized.

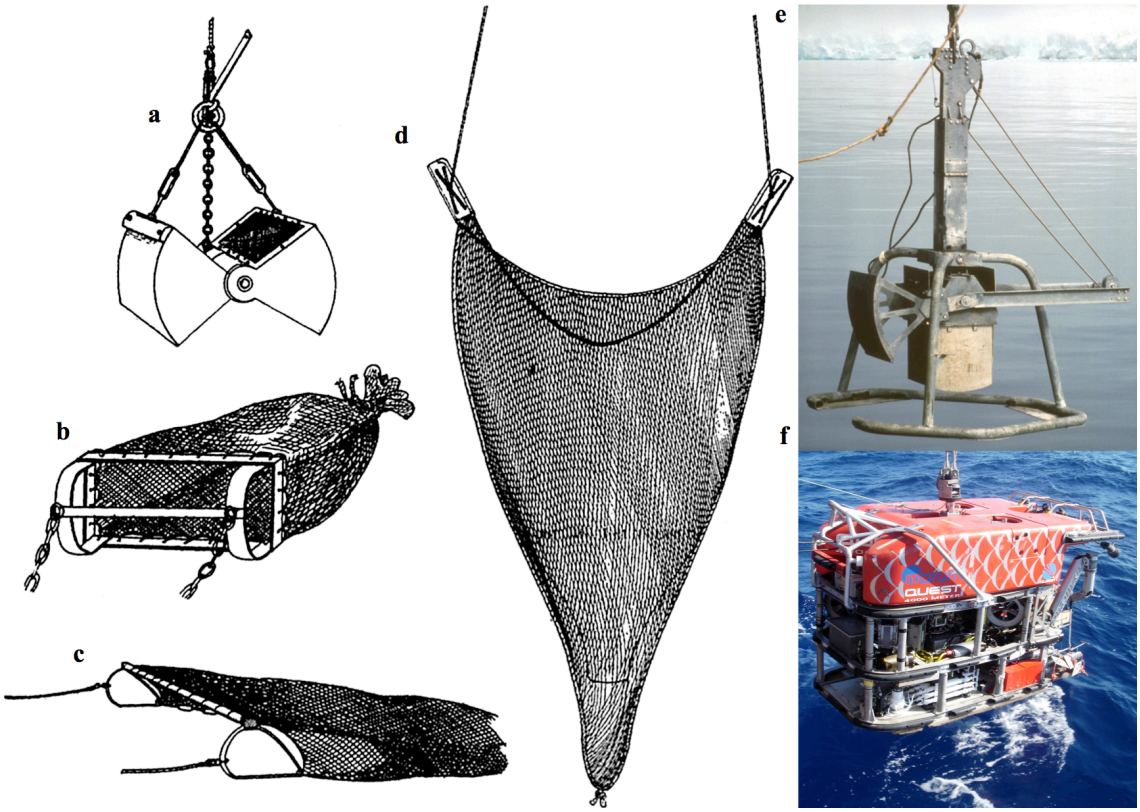


Figure 1. Gear for biological collecting: *a*, Petersen grab; *b*, dredge; *c*, beam-trawl; *d*, otter-trawl (Sverdrup *et al.* 1942); *e*, box corer; *f*, Remote Operated Vehicle (QUEST4000 (MARUM), www.mpi-bremen.de)

All cruises were conducted in compliance with local legislations and according to the Convention on Biological Diversity (CBD); samples were transported to the Museo Nacional de Ciencias Naturales (MNCN) with appropriate export and import permits following the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) regulations.

Detecting and Measuring Differentiation Analyses

The analyses performed in this Thesis in order to detect and measure variability of *D. dianthus* at morphological and molecular levels, are following:

Morphology: corallum and cnidom

Morphologic characters used to characterize corallum and cnidom of *D. dianthus* at inter and intraspecific level were stored in discrete and continuous data matrices.

Morphometric variables were tested for normality (Shapiro-Wilk's W and Kolmogorov-Smirnov tests) and log-transformed when necessary, for equality of means (Student's t-test) and variances (Levene's test), as well as subjected to the analysis of variance.

Parametric ANOVAs were performed for the variables meeting the assumptions of normality and homogeneity of variance. Tukey tests (Multivariate test- with Wilks' Lambda) for significant differences for unequal samples were used to perform the post-hoc comparisons of means. Non-parametric Kolmogorov-Smirnov (for two groups) and Kruskal-Wallis tests for the analysis of variance were used for variables, which failed to meet the ANOVA assumptions.

Morphological characters were subjected to Canonical Discriminant Analyses (CDAs). The analyses were performed using several categories as a priori groups. Correlations between discriminant functions and initial variables were examined.

Landmarks coordinates were first used to construct wireframes (i.e. sets of straight lines (wires) connecting selected landmarks on a shape) employed for visualization in further analyses and could also be used to calculate the length of each wire (and thus the distance between certain landmarks). Subsequently, all information not related to shape, including size, from the analysis was removed by minimizing the distance between the landmarks in different configurations through a series of translation, scaling and rotation (Procustes superimposition method). The output partial wrap scores were subjected to Principal component analyses (PCAs) and canonical variate analyses (CVAs), in order to determine the axes with the most variation among specimens and between predetermined groups, respectively (Zeldtich *et al.* 2004).

Multivariate analyses of variance (MANOVAs) of data were also performed. MANOVA is a statistical test of for assessing differences among groups that tests the null hypothesis that there is no significant differences.

Molecular: gene and genomics, phylogeny, and population genetics

Nuclear and mitochondrial genes with distinct parameters, such as evolution rates, polymorphism level, expression type and heredity mode, were used as molecular markers to characterize *D. dianthus'* genetic variation at inter and intraspecific level.

Genetic flowchart involved five steps: the DNA extraction of samples, the PCR (Polymerase Chain Reaction) amplifications of genes, the sequencing (genotyping) of PCR products, the alignment of sequences (alleles assignment) and the analyses of data set.

Genomics pipeline involved three main steps: the sequencing of DNA by high-throughput technologies (such as Illumina and 454 pyrosequencing), the assembling of reads to scaffolds, relative quality control and the annotation of sequences.

Phylogenetic Inference

Phylogeny is the evolutionary history of a species, based on Darwin`s theory of descendant with modification: similar traits in different organisms and inherited from a common ancestor, are evidence of genealogic relationship. From an evolutionary point of view, similarity can be divided in two types: homology and analogy. Homology is any similarity between characters that is due to their shared ancestor, whereas analogy occurs when characters are similar but do not derived from a common ancestor; thus parallelism, convergence or reversal lead to homoplastic characters (Simpson, 1961).

The hurdle is based on the fact that similarity is not given, but it is interpreted. Different interpretation criteria led different methods of phylogenetic analysis, and consequent controversy among biologists (Caballero and Suárez 1999).

Phenetic systematists (Sokal and Sneath 1963) attempt to interpret relationships among taxa based on overall similarity calculated by numerical methods (such as distance matrices, and similarity indices of genetic divergences). Phenetic analyses do not distinguish between homologous and homoplastic character states.

Evolutionary systematists (Simpson 1961; Mayr 1969) state that only similarities based in homology can be used to interpret phylogenetic relationships among taxa.

Phylogenetic and cladistic systematists (Hennig 1950,1966; Eldredge and Cracarft 1980; Nelson and Platnick 1981; Wiley 1981) distinguish between plesiomorphies (i.e. ancestral or primitive traits that are inherited from an ancestor and therefore phylogenetically uninformative), and apomorphies (i.e. derived traits of a clade, therefore phylogenetically informative). Thus, only synapomorphies (i.e. shared derived

character states between two o more taxa or lineages and their most recent shared ancestor) of different taxa are evidences of close relationship and, consequently, define monophyletic lineages.

Although it is not strictly a phylogenetic method, Distance Methods (Neighbour-Joining, NJ, and Unweighted Pair Group Method using Arithmetic Averages, UPGMA) were commonly used to recreate topology based on genetic similarity. The main methods used to infer phylogenetic trees (topologies) are Maximum Parsimony (MP), Maximum Likelihood (ML) and Bayesian Inference (BI), and three types of classification can categorize them on the basis of their overall framework. First of all, they can be grouped in two categories based on data management: method that treat data sets in a combination of continuous characters, such as a distance matrices (NJ, UPGMA), and those that consider data sets as a combination of discrete characters (MP, ML, and BI). Secondly, the methods can be grouped in two categories based on phylogenetic criterion: methods that apply data clustering algorithms to obtain topologies (NJ, UPGMA), or methods that use data searching algorithms based on optimization criteria to obtain the best topology (MP, ML, and BI). And finally, the methods can be grouped in two categories based on the implementation of evolution model: methods that do not apply models describing the evolution of characters observed in the species (MP), and methods that consider evolution models as a parameter for phylogenetic reconstruction (NJ, ML, BI).

- **Maximum Parsimony** (MP) methods are based on the principle of Occam's Razor: lacking any other factors, the simplest explanation (the most parsimonious one) should be chosen. Given a dataset, possible phylogenetic trees representing alternative relationships among the OTUs (Operational Taxonomic Units) are compared and each one is given a score that reflects the minimum number of character state changes (e.g., amino acid substitutions) that would be required over evolutionary time to fit the sequences into that tree. The optimal tree (the most parsimonious) is considered to be the one requiring the fewest changes (Eisen 1998).

- **Distance** (NJ, UPGMA) methods use the evolutionary distances (i.e. distance matrices) between OTUs to infer phylogenetic history. The optimal tree is generated by first calculating the estimated evolutionary distances between all pairs of sequences.

These distances are then used to generate a tree in which the branch patterns and lengths best represent the distance matrix. For such methods, corrections are essential to convert measures of similarity to evolutionary distance (Eisen 1998).

One limitation of both the parsimony and distance methods is that although they may select one tree over another on the basis of some criteria, it is not possible to say how much more probable one tree is than another. Likelihood and Bayesian methods have been designed to provide such statistical framework for phylogenetic reconstruction.

- **Maximum Likelihood** (ML) methods consider the phylogenetic inference as a statistical question. The method is similar to parsimony methods in that possible trees are compared and given a score. They differ significantly in that their tree scores are based on the probability (likelihood) for a given phylogenetic tree (topology) of producing the observed data set (sequences), assuming a particular evolution model (model of amino acid or nucleotide substitution probabilities). The optimal tree is considered to be the one that has the highest probability.

- **Bayesian Inference** (IB) methods are a variant of ML in which the likelihood of a tree itself (posterior probability) is calculated and assigned after *a priori* probabilities are taken into account. Thus, rather than trying to calculate the probability that a particular hypothesis could generate the data, Bayesian methods seek to calculate the actual probability of the hypothesis by attempting to assign a value to the prior odds (i.e. the probability of the data and the probability of the hypothesis). The trees scores are based on the probability (likelihood) for the phylogenetic tree (topology) to have fitted in a given data set (sequences, morphological characters) and an evolutionary model (amino acid or nucleotide substitutions, and morphological changes probabilities). The optimal tree is considered to be the one that has the highest posterior probability.

Population genetics

The field of population genetics, whose the focus is the population or the species and not the individual, came into being in the 1920s and 1930s, thanks to the works of R. A. Fisher, J. B. S. Haldane and S. Wright as a vital ingredient in the emergence of the modern evolutionary synthesis (Fisher 1918; Haldane 1924; Wright 1932). Population genetics investigates the structure of genetic variation across space (geographic

variation) and time (evolutionary change), analyzing the frequency and the changes in frequencies of occurrence of alleles and genotypes within and between populations. And because changes in gene frequencies are at the heart of evolution and speciation, population and evolutionary genetics are often studied together.

The alleles are different versions of the same gene that are expressed as different phenotypes, and their appearance is based on the random and natural process of mutation, whereas the frequency of occurrence of an allele changes regularly as a result of mutation, genetic drift, recombination and selection. Since a population needs variation to best adapt to a changing environmental the measure of the amount of heterozygosity across all genes can be used as a general indicator of the amount of genetic variability and genetic health of a population. Furthermore, the population's structure affects the extent of genetic variation and its patterns of distribution; also a population is considered structured if genetic drift is occurring in some of its subpopulations, migration does not happen uniformly throughout the population or mating is not random throughout the population.

Mathematical models are used to investigate and predict the occurrence of specific alleles or combinations of alleles in populations based on developments in the molecular understanding of genetics, Mendel's laws of inheritance and modern evolutionary theory. Several statistical measures of population genetics are performed in order to elucidate the individual and population's genetic variability, structure and dynamics, and are presented as follows:

- **Test for Hardy-Weinberg Equilibrium** (HWE): Populations in their natural environment can never meet all of the conditions required to achieve Hardy-Weinberg equilibrium (i.e. in a large, randomly breeding (diploid) population, allelic frequencies will remain the same from generation to generation, assuming no unbalanced mutation, gene migration, selection or genetic drift); thus their allele frequencies will change from one generation to the next and the population will evolve. How far the population deviates from Hardy-Weinberg is an indication of the intensity of external factors, and can be determined by a statistical formula (chi-square), which is used to compare observed (Ho) versus expected (He) heterozygosity.

- **Effective Population Size**: genetic drift, the random increase or decrease of an allele's frequency, affects small populations more severely than large ones, since alleles are drawn from a smaller parental gene pool. One of the many variables of population dynamics that can influence the rate and size of fluctuation in allele frequencies is the population size. Thus, the rate of change in allele frequencies in a population is determined by the population's effective population size, which is the number of individuals that evenly contribute to the gene pool.

- **Inbreeding and Relatedness**: small effective population size can result in a high occurrence of inbreeding, or mating between close relatives. One of the effects of inbreeding is a decrease in the heterozygosity (increase in homozygosity) of the whole population, which means a decrease in the number of heterozygous genes in the individuals. This effect could place individuals and the population at a greater risk, due to, for instance, homozygous recessive diseases that result from inheriting a copy of the same recessive allele from both parents and consequently the loss in population vigour due to loss in genetic variability or genetic options (inbreeding depression).

If it is assumed that genotypes in a population are in Hardy-Weinberg assumptions, a set of statistical indices (F statistics or fixation indices, Sewell Wright 1950s) describes the expected level of heterozygosity within the population, measuring the probability that two alleles in an individual are identical by descent relative to 1) the subpopulation from which they are drawn (F_{IS}), 2) the subpopulation respect to the total (F_{ST}), and 3) the entire population (F_{IT}). In other words, the F-statistics can specifically measure the expected degree of reduction in heterozigosity, indicating the deficiency or excess of average heterozygotes within each population (F_{IS}), the degree of gene differentiation among populations in terms of allele frequencies (F_{ST}), and the deficiency or excess of average heterozygotes in a group of populations (F_{IT}).

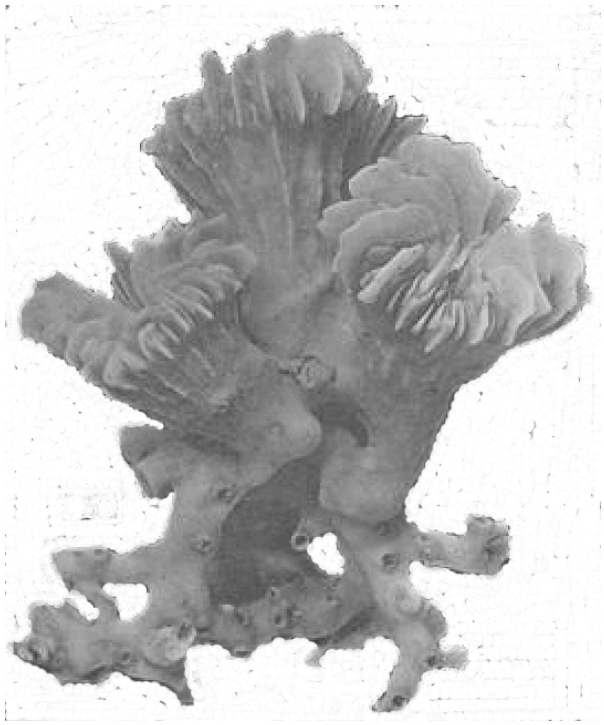
- **Cluster methodology**: Bayesian statistics is used to classify groups of individuals based on their genetic similarity. Assuming that populations are in HWE, each specimen is assigned with a specific probability to a group or 'cluster' (K) based on allelic frequencies of their genotypes. The cluster Bayesian methodology presents a relevant limitation that it assumes existing *a priori* populations, which do not always correspond to biological units (Pearse and Crandall 2004).

Modern computational approaches, often using coalescent theory, have played a central role since the 1980s. The prospective (classical) population genetics predict changes in the frequencies of alleles forward in time and to describe patterns of genetic variation in an entire population. On the contrary, the retrospective approach (coalescence) demonstrates that a relatively simple ancestral process for a sample exists and obviates the need of explicitly modelling the entire population.

- **Coalescent theory**: describes the connection between demographic history and genetic data, providing a framework to extract information from samples of DNA sequences. The coalescence describes the genetic ancestry of a sample (i.e., gene genealogy) and uses it to make predictions about patterns of genetic variation. Hudson (1983) and Tajima (1983) explored the coalescence under different biological scenarios and presented more intuitive derivations such as the most commonly-used population model: the Wright-Fisher model. Next to Mendel's Laws, the coalescence may be the best justified and farthest reaching stochastic mathematical model in biology, with a broad utility: molecular ecology, phylogeography or evolutionary genetics (Kingman 1982).

RESULTS

CHAPTER I



Chapter adapted from:

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Desmophyllum crista galli. Charles Joseph Gravier (1920). Madreporaires provenant des Campagnes des yachts Princesse-Alice et Hirondelle II (1893-1913).

Desmophyllum dianthus **(Esper, 1794) in the scleractinian phylogeny and its intraspecific diversity.**

Abstract

The cosmopolitan solitary deep-water scleractinian coral *Desmophyllum dianthu*s (Esper, 1794) was selected as a representative model species of the polyphyletic Caryophylliidae family to (1) examine phylogenetic relationships with respect to the principal Scleractinia taxa, (2) check population structure, (3) test the widespread connectivity hypothesis and (4) assess the utility of different nuclear and mitochondrial markers currently in use. To carry out these goals, DNA sequence data from nuclear (ITS and 28S) and mitochondrial (16S and COI) markers were analyzed for several coral species and for Mediterranean populations of *D. dianthus*. Three phylogenetic methodologies (ML, MP and BI), based on data from the four molecular markers, all supported *D. dianthus* as clearly belonging to the "robust" clade, in which the species *Lophelia pertusa* and *D. dianthus* not only grouped together, but also shared haplotypes for some DNA markers. Molecular results also showed shared haplotypes among *D. dianthus* populations distributed in regions separated by several thousands of kilometers and by clear geographic barriers. These results could reflect limited molecular and morphological taxonomic resolution rather than real widespread connectivity. Additional studies are needed in order to find molecular markers and morphological features able to disentangle the complex phylogenetic relationship in the Order Scleractinia and to differentiate isolated populations, thus avoiding the homoplasy found in some morphological characters that are still considered in the literature.

Keywords: cold-water coral; *Desmophyllum dianthus*; Mediterranean Sea; nuclear markers; mitochondrial markers; Systematics.

Introduction

Deep-sea ecosystems represent the largest biome of the global biosphere (Gage and Tyler 1991; Danovaro *et al.* 2010) in which cold (or deep) water corals (CWC) play a significant ecological role (Roberts *et al.* 2006). In spite of this, many fundamental traits of cold-water coral biology still need to be more properly understood. One such deficiency is in the scantiness of studies focusing on the molecular biology of CWCs, which would shed light on their proper supraspecific taxonomic positioning, phylogeography and connectivity.

When morphological features are insufficient to disentangle the evolutionary history of certain taxa, molecular phylogenies (inter- and intraspecific) can provide evidence of past evolutionary events, and allow comparisons of intra- and interpopulation variability to identify patterns of biological diversity (Trewick and Wallis 2001; Knowles and Maddison 2002; Calvo *et al.* 2009). Recently, multidisciplinary approaches have played a strong role in scleractinian systematics (Budd *et al.* 2010) (Stefani *et al.* 2008), but the results of these efforts are not uniform, especially for solitary azooxanthellate CWCs, thereby causing a biased view of the evolutionary history and global biogeography of Scleractinia (Lindner *et al.* 2008; Stefani *et al.* 2008; Barbeitos *et al.* 2010; Kitahara *et al.* 2010b; Stolarski *et al.* 2011).

Additional molecular data for more solitary CWC could corroborate the concept that some CWC species are widely distributed. However, recent studies have shown that some of these widespread eurybathic species actually represent multiple genetically distinct cryptic species that can be subdivided by geography or depth (Carlon and Budd 2002; Raupach *et al.* 2007; Brandão *et al.* 2010).

CWC are widespread in the Mediterranean Sea, occurring as either extant species or as Pleistocene fossils (Zibrowius 1980; Freiwald *et al.* 2009; Taviani *et al.* 2011b). For many years, CWC were considered to be near extinction in the Mediterranean Sea until the unexpected rediscovery of living banks of *Lophelia pertusa* and *Madrepora oculata* in Santa Maria di Leuca, in the Ionian Sea (Mastrototaro *et al.* 2002). More discoveries of these species followed, with new sites found in the southwestern Adriatic Sea, Strait of Sicily, Catalan-Provencal margin and Alboran Sea (Schembri *et al.* 2007; Trincardi *et al.* 2007; Freiwald *et al.* 2009; Orejas *et al.* 2009; Fink *et al.* 2012).

The current interest in Mediterranean CWC ecosystems necessitates an assessment of the biological status of its major coral components, among which the solitary scleractinian *Desmophyllum dianthus* occupies an important position.

Desmophyllum dianthus (Esper, 1794) (syn. *Desmophyllum cristagalli*, according to Cairns and Zibrowius (1997) is a species still considered as cosmopolitan, and specimens have been reported in all oceans of the world from coastal Antarctic to the Arctic Circle. It is a solitary coral, classified in the family Caryophylliidae based on morphological characters. *Desmophyllum dianthus* is a slow-growing coral (0.5-2 mm per year) with a long lifespan (up to 200 years) (Risk *et al.* 2002; Adkins *et al.* 2004).

Desmophyllum dianthus occurs in the upper bathyal zone (common depth range between 200-2,500 m; (Zibrowius 1980; Roberts *et al.* 2009)), associated with deep-water coral reefs frame building species (e.g. *Lophelia pertusa* and *Madrepora oculata*). However, records at shallower depths exist for New Zealand fjords -from 20 m, (Grange *et al.* 1981) - and Chilean fjords -from 8 m, (Försterra and Häussermann 2003) -, where *D. dianthus* was found in an unusual symbiosis with the microendolithic phototrophic alga *Ostrobium quecketii* (Försterra and Häussermann 2008).

Desmophyllum dianthus also contributes to the reef framework as aggregated colonies or "clumps of specimens" (Cairns 1982). This species is a preferred target to study oceanographic-climatic variability by deciphering geochemical signals embedded within its skeletal aragonite (Sorauf and Jell 1977; Risk *et al.* 2002; Adkins *et al.* 2004; Boyle 2006; Montagna *et al.* 2006). Recently, preliminary analyses of the phylogenetics, ecology, including the unusual symbiosis with algae, and reproduction (Försterra and Häussermann 2003; Försterra and Häussermann 2008; Miller *et al.* 2010; Miller *et al.* 2011; Stolarski *et al.* 2011) have been conducted for *D. dianthus*, but each topic still needs further investigation to gain comprehensive knowledge about this species. Here, we aim to 1) characterize Mediterranean *D. dianthus* with molecular markers and investigate its phylogenetic relationships with respect to principal scleractinian taxa; 2) undertake the study of the genetic structure of extant populations; 3) validate the widespread connectivity hypothesis and 4) corroborate the utility of nuclear (ITS1-5.8S rRNA-ITS2 and 28S rRNA) and mitochondrial (16S rRNA and COI) markers.

Material and Methods

Samples collection, species and study area

Live *D. dianthus* specimens were collected from the Mediterranean Sea at depths between 276-1,102 m from living CWC grounds in the Adriatic Sea, Ionian Sea and Strait of Sicily (Figure 1.1, Table 1.1). Specimens analyzed in this study were initially preserved in 80% ethanol at 4ºC prior to being stored in absolute ethanol.

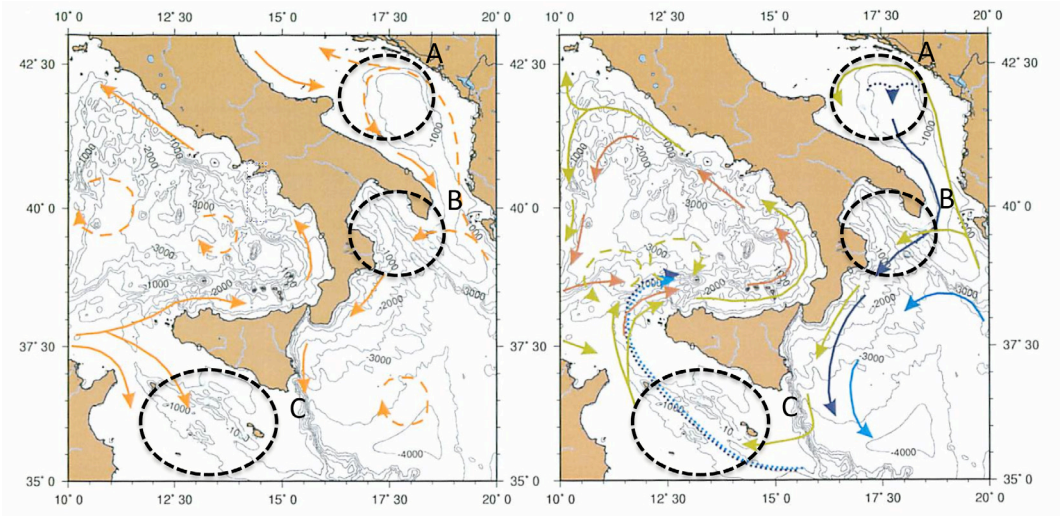

Figure 1.1. Water masses and circulation in the central Mediterranean Sea, modified from Reithdorf (2008). The black circles represent the sampling sites in the central of Mediterranean Sea: Adriatic Sea (A), Ionian Sea (B) and Strait of Sicily (C).

This study was based on specimens collected in 2006/2007 and 2009, during scientific cruises M70, SETE06, APLABES, CORSARO, CORAL2002, MARCOS and MEDCOR on board the RV *Meteor* and RV *Urania*. All necessary permits were obtained for the described field studies. The study areas were not marine protected or privately owned areas, and sampling did not require any specific permission. This study did not involve endangered or protected species listed in the IUCN Red List of Threatened Species.

Table 1.1. Depth, method, and geographic coordinates of sampling locations.

Area	Proyect	Year	Campaign	Cruise Code	Site	Station nº	Lat. N (start)	Long. E (start)	Depth (start)	Lat. N (end)	Long. E (end)	Depth (end)	Technique	*Dd* Code
A: Adriatic Sea	HERMES	2006	M70/3	M70	Bari Canyon	735	41°17'28.62"	17°16'37.38"	664	41°16'58.56"	17°16'34.44"	276	ROV Quest 4000	DdBAR
	HERMES	2006	M70/6	M70	Bari Canyon	745	41°17'49.5"	17°16'37.38"	557	41°17'31.92"	17°09'57.03"	315	ROV Quest 4000	DdBAR
	HERMES	2006	M70/4	M70	Dauno Seamount	739	41°33'1.47"	17°28'5.91"					ROV Quest 4000	DdDAU
	HERMES	2006	M70/5	M70	Gondola Slide	752	41°43'30.48"	17°02'47.64"	710	41°43'10.62"	17°03'39.3"	674	ROV Quest 4000	DdGON
	HERMES	2006	SETE06	SE06-48DR	off Gargano		41°72'11.28"	17°05'39.3"	728	41°71'49.86"	17°06'15.78"	704	Epibenthic sledge	DdGAR
B: Ionian Sea	APLABES	2005	APLABES	AP01	Santa Maria di Leuca		39°34'50.4"		513	18°23'18"		513	Grab	DdSML
	APLABES	2005	APLABES	AP30	Santa Maria di Leuca		39°28'5.4"		747	18°24'25.2"		747	Grab	DdSML
	HERMES	2006	CORSARO	CR37	Santa Maria di Leuca		39°33'14"	18°13'17"	548	39°33'29"	18°13'08"	538	Epibenthic dredge	DdSML
	HERMES	2006	CORSARO	CR39	Santa Maria di Leuca		39°33'14.8"	18°13'16.3"	577	39°33'27.4"	18°13'111"	540	Epibenthic dredge	DdSML
	HERMES	2006	CORSARO	CR55	Santa Maria di Leuca		39°34'55.5"	18°23'21.7"	501	39°35'20.9"	18°23'39"	497	Epibenthic dredge	DdSML
	HERMES	2006	CORSARO	CR72	Santa Maria di Leuca		39°37'19"	18°38'59"	678	39°36'43"	18°38'44"	701	Epibenthic dredge	DdSML
	HERMES	2006	CORSARO	CR73	Santa Maria di Leuca		39°37'29"	18°39'05"	671	39°38'07"	18°40'23"	679	Agassiz trawl	DdSML
	HERMIONE	2009	CORAL2002	COR2-111s	Santa Maria di Leuca		39°35'22.64"	18°22'59.99"	497	39°35'44.88"	18°22'46.44"	482	Epibenthic trawl	DdSML
	HERMES	2006	CORSARO	CR22	off Gargano		39°50'00"	17°37'45"	1102	39°50'26"	17°38'01"	985	Rock dredge	DdGAL
	HERMES	2006	CORSARO	CR26	off Gargano		39°49'56.5"	17°37'45.4"	1097	39°50'38"	17°37'30"	1010	Rock dredge	DdGAL
	HERMES	2006	M70/2	M70	Gallipoli Escarpment	708	39°37'18.35"	18°04'47.51"	823	39°37'30.66"	18°05'1.08"	574	ROV Quest 4000	DdGAL
	-	2006	Local boat	RI	Roccella Ionica		38°18'2"	16°29'8"	-				Lost crab trap recovery	DdROC
C:Strait of Sicily	HERMES	2006	M70/1	M70	Urania Bank	677	36°50'19.56"	13°09'20.03"	654	36°50'16.44"	13°09'15"	440	ROV Quest 4000	DdURA
	HERMES	2007	MARCOS	MS22	Urania Bank		36°50'20.46"	13°09'21.72"	626				Grab	DdURA
	HERMES	2007	MARCOS	MS36	Linosa		35°46'0.6"	13°02'36.54"	819	35°45'46.74"	13°02'19.02"	403	Epibenthic trawl	DdLIN
	HERMES	2007	MARCOS	MS43	Malta	25	35°30'43.2"	14°06'33.66"	607	35°30'48.18"	14°06'30.66"	452	Epibenthic trawl	DdMAL
	HERMIONE	2009	MEDCOR	MEDCOR25	Malta	25	35°30'28.66"	14°11'2.74"	690	35°31'0.98"	14°10'26.034"	462	Epibenthic minidredge	DdMAL
	HERMIONE	2009	MEDCOR	MEDCOR74	Gela Basin	74	36°45'23.292"	14°00'6.21"	824	36°44'18.42"	13°58'28.84"	850	Epibenthic minidredge	DdGEL

DNA extraction, PCR amplification and sequencing

Small pieces of tissue were taken from each sample and rinsed with ultrapure water prior to extraction. DNA extraction was performed using the QIAGEN BioSprint 15 DNA Blood Kit (Qiagen Iberia S.L., Madrid) following the manufacturer's instructions, but with an extended period of proteinase K lysis (overnight incubation at 55 °C).

For each specimen, the concentration of extracted genomic DNA was measured using a Nanodrop 1000 (Thermo Scientific). Each aliquot was then diluted at a ratio of 1:20 in 200 μl final volume.

Four partial DNA genes and regions, including nuclear and mitochondrial markers with different rates of mutation, were partially amplified and sequenced: 1) the internal transcribed spacer regions (internal transcribed spacer 1-5.8S ribosomal DNA - internal transcribed spacer 2, hereafter designated ITS), 2) the large ribosomal subunit (28S), 3) the mitochondrial large ribosomal subunit (16S) and 4) the mitochondrial cytochrome oxidase c subunit I (COI).

- Nuclear genes

Polymerase chain reactions (PCR) were carried out in a total volume of 50 μl, with 1x PCR Buffer (final concentration $MgCl_2$ 2mM), up to 3mM $MgCl_2$ (only for 28S), 0.05 mM of each dNTP, 0.14 μM of each primer, 1.5U of Taq polymerase and 2 μl of template DNA.

Nuclear ITS was amplified using the primers ITS2.1 and ITS2.2 (Hugall *et al.* 1999), and a portion of the 5' end of the nuclear 28S (including the C1 and D2 domains) was amplified using the primers C1' and D2MAD (Cuif *et al.* 2003). PCR amplification was performed on extracted DNA under the following conditions: an initial denaturing step of 4 min at 94 ºC, followed by 40 cycles of 45 s (ITS) or 1 min (28S) at 94 ºC, 1 min annealing at 57 ºC (ITS) or 56 ºC (28S), and 1 min extension at 72 ºC and a final extension step of 10 min at 72 ºC.

The products were visualized under blue light in a 1.5% agarose gel stained with SYBR Safe, and then purified using an ethanol/sodium acetate precipitation method. Both strands were sequenced using BigDye Terminator and an ABI PRISM 3730 DNA Sequencer (Applied Biosystems).

- Mitochondrial genes

PCR reactions were carried out in 20 μl using the HotStartTaq Plus Master Mix Kit (QIAGEN), following the manufacturer's instructions, and with 1 μl of each primer (10 μM each) and 1 μl of template DNA.

16S was amplified using the hard coral specific primers LP16SF and LP16SR (Le Goff-Vitry *et al.* 2004b).

COI was amplified using a novel forward primer COIcoralF (5'-GATCATCTTTATAATTGT-3') and the reverse primer HCO2 (Folmer *et al.* 1994). The COIcoralF primer was specifically designed for Scleractinia with Corallimorpharia sequences as outgroup. The following thermal cycle conditions were utilized: an initial activation step of 5 min at 95 ºC, followed by 30 cycles of 30 s at 94 ºC, 30 s at 50 ºC, and 45 s at 72 ºC with a final extension step of 10 min at 72 ºC.

Amplified products were cleaned up by Exo1/SAP treatment, with the following thermal cycling conditions: 20 min at 37 ºC, 30 min at 83 ºC, and a final hold at 4 ºC. Both strands were sequenced using BigDye Terminator and an ABI3730XL DNA Sequencer (Applied Biosystems).

For both nuclear and mitochondrial markers, when amplification failed, different dilutions of template DNA up to 1:500 were used to repeat the PCR.

- Alignments

Sequences were verified and primers were cut from the alignment using the program Sequencher v4.10.1 (Gene Codes Corporation). In order to expand our result to a wide spectrum of families in Scleractinia with special emphasis on Caryophylliidae, we also sequenced specimens for potentially closely related species. Additional previously published nuclear and mitochondrial sequences were also retrieved from GenBank and added to the alignments (Table 1.2).

The nucleotide sequences of ITS, 28S, 16S and COI were separately aligned in ClustalX (Thompson *et al.* 1997) using the default settings. The resulting alignments were inspected by eye and manually checked and adjusted with Se-Al v2.0a11 (Rambaut 2002). Consequently, four matrices with the final alignments were generated.

Phylogenetic analyses

The model of best fits for nucleotide evolution for each final alignment was determined by the Akaike Information Criterion (AIC) in jModelTest (Posada 2008). Phylogenetic analyses were performed using PhyML v3.0 (Guindon and Gascuel 2003) for Maximum Likelihood (ML), MrBayes v3.1.2 (Ronquist and Huelsenbeck 2003) for Bayesian Inference (BI), and PAUP*v4.0b10 (Swofford 2002) for Maximum Parsimony (MP). The different data sets were analyzed separately and then tested for heterogeneity with PAUP*v4.0b10 between data partitions, before combining the data in a unique matrix. The ML and MP analyses were performed with 1000 bootstrap replicates. For the BI analyses, five double parallel runs were performed for 5 million generations with one cold and three heated Markov Chains Monte Carlo (MCMC) for each run, sampling trees at 1000 generations intervals (5000 trees were saved during MCMC for each run) when the average standard deviation of split frequencies betweens runs was less than 0.01. The addition of more generations (up to 10 million) was necessary for some matrices to reach a standard deviation of split frequencies below 0.01 and for the effective sample size (ESS) to reach the suggested minimum value (>200), as verified using Tracer v1.5 (Rambaut and Drummond 2009). Ten percent of all trees were discarded as burn-in, and the remaining trees were used to calculate the posterior probabilities. Maximum clade credibility trees were generated by TreeAnnotator (Drummond and Rambaut 2007).

To place *D. dianthus* within one of the Caryophylliidae clades as defined by previous studies (Romano and Cairns 2000; Cuif *et al.* 2003; Kerr 2005; Kitahara *et al.* 2010b), nuclear (ITS, 28S) and mitochondrial (16S, COI) sequences were obtained from this study. Additional sequences were downloaded from GenBank (Annexe 1) for the following caryophylliid genera: *Caryophyllia, Crispatotrochus, Deltocyathus*, *Lophelia, Polycyathus*, *Paracyathus*, *Cladocora*, *Rhizosmilia*, *Phyllangia, Ceratotrochus*, *Odontocyathus, Vaughanella, Thalamophyllia*, *Tethocyathus, Solenosmilia, Pourtalosmilia, Stephanocyathus, Trochocyathus, Conotrochus, Dactylotrochus* and *Dasmosmilia.* Scleractinia genera were recognized based on classification assigned by Cairns and listed in Roberts *et al.* 2009. Taxonomic discrepancies of some genera (e.g. *Cladocora*) are not included in this study. Representative species of the following families were also included in the analyses: Flabellidae, Dendrophylliidae, Poritiidae,

Siderastreidae, Acroporidae and Agariciidae ('complex' corals); Meruliniidae, Pectiniidae, Faviidae, Mussiidae, Pocilloporiidae and Fungiidae ('robust' corals); Meandriniidae, Oculinidae, Astrocoeniidae and Euphylliidae (families with species that can be included in both 'complex' and 'robust' groups); Micrabaciidae and Gardineriidae ('basal' corals as defined by Kitahara *et al.* 2010 and Stolarski *et al.* 2011). *Ricordea florida* (Anthozoa: Corallimorpharia) was selected as the outgroup species for all analyses.

Haplotype network

Intraspecific phylogenies were evaluated using a network approach. Analyses were performed on 54 *D. dianthus* specimens analyzed for ITS haplotypes (551 bp) and 49 for 16S haplotypes (281 bp) from two geographic regions, the South Pacific Ocean and the Mediterranean Sea. A median-joining network was performed with the software Network 4.610 (Fluxus Technology Ltd), based on ITS and 16S alignments, as they are the only *D. dianthus* sequences well represented in GenBank. The framework for testing evolutionary hypotheses was obtained using neutrality test, genetic polymorphism and gene flow analyses, performed with DnaSP v.5.0 software (Librado and Rozas 2009) and Arlequin v3.5.1.2 (Excoffier *et al.* 2005).

Results

Four alignments were obtained: 1) ITS (112 taxa), 2) 28S (98 taxa), 3) 16S (57 taxa) and 4) COI (51 taxa) (Table 2).

Table 1.2. Length of PCR products and respective alignments of *Desmophyllum dianthus* and the main scleractinian family taxa (for more details see Annexe 1), along with the best-fit models selected by AIC in Modeltest 3.7. *Considering gaps as a fifth state of characters.

	ITS	**28S**	**16S**	**COI**	**Combined**
PCR product length	610	759	280	538	-
Alignment length	843	791	475	538	2578
Variable characters	426 (*551)	175 (*344)	163 (*327)	194	965 (*1425)
Parsimony informative characters	239 (*393)	95 (*177)	108 (*266)	153	595 (*1001)
Model of best fit	TIMef+G	GTR+I+G	TrN+G	HKY+G	TIM+I+G

Taking nuclear (ITS + 28S) and mitochondrial (16S + COI) data together, a total of 2,595 sites from 44 specimens were analyzed for the four DNA regions utilized in this

study. New sequences obtained in the present study were deposited in GenBank (Annexe 1).

Phylogenetic analyses

The results of phylogenetic analyses under BI, ML and MP approaches are summarized in Fig. 1.2, Fig. 1.3 and Fig. 1.4. Phylogenetic analyses of each gene region were consistent and yielded the same tree topologies. Nuclear and mitochondrial DNA combined analyses provided the greatest resolution, after verifying their congruence through partition-homogeneity test (P= 0.26). The phylogenetic analyses indicated *D. dianthus* within the well-supported monophyletic "robust" group, consistent with previous analyses. No apparent genetic structure was found for *D. dianthus* sequences in any of the phylogenetic analyses, and the sequences consistently appeared as a polytomy (Fig. 1.2). Almost no inter-individual divergence was found among the sequences of this species, with a maximum of 0.93% divergence for the combined matrix. The closest related species to *D. dianthus* was *L. pertusa*, showing 2.25% and 4.80% maximum pairwise divergence with *D. dianthus* for mitochondrial DNA sequences (COI and 16S, respectively) and 0.26% and 2.10% for nuclear regions (28S and ITS, respectively); but no noteworthy divergences among most sequences of both species were found in 28S, ITS, and 16S data. In fact, the *D. dianthus* + *L. pertusa* clade was always highly supported by both bootstrap values and posterior probabilities. The phylogenetic relationships based on all utilized markers, as shown in Fig. 1.2, also fully supported *Caryophyllia calveri* as the sister group to *D. dianthus* + *L. pertusa*, with a high range of pairwise divergence values (1.98 and 9.25% for 28S and COI, respectively). *Caryophyllia smithii* and *Pourtalosmilia anthophyllites* completed the Caryophylliidae family cluster, but it could not be considered a clade as the position of *Cladocora caespitosa* was unresolved in the different phylogenetic analyses. *Montastrea faveolata* + *Mussa angulosa*, *Madracis mirabilis,* and *Madrepora oculata* completed the representative species of the scleractinian "robust" group. Depending on what gene was selected, the "complex" group appeared to be monophyletic or paraphyletic. Caryophylliidae species, which clustered in both groups, were not recovered as monophylies at the genus level or into putative clades as defined by previous studies (Kerr 2005; Kitahara *et al.* 2010b).

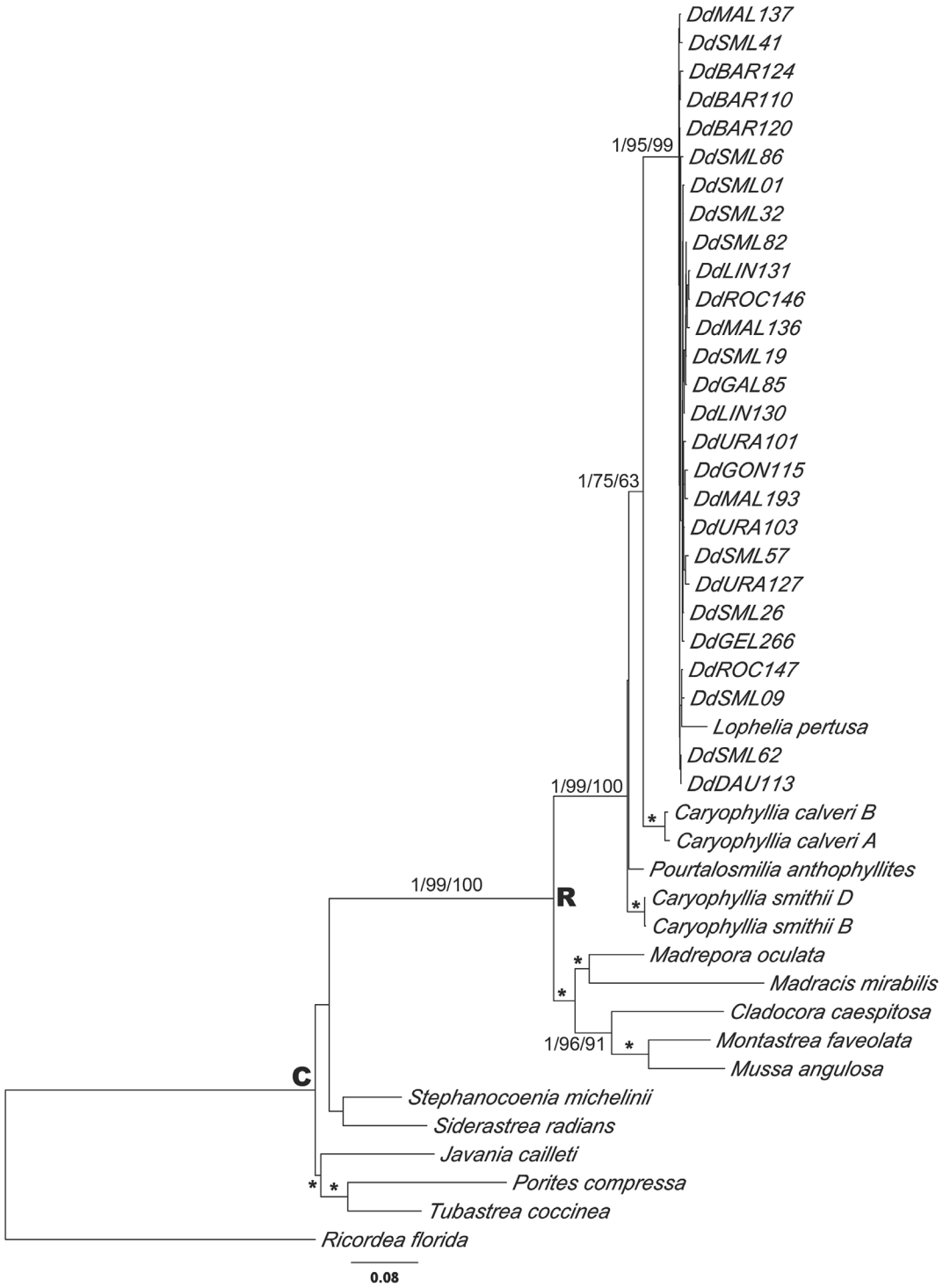

Figure 1.2. Phylogenetic relationship between *Desmophyllum dianthus* and principal taxa from scleractinian families. Tree topology was inferred by Bayesian analysis, based on combined mitochondrial and nuclear genes. R and C indicate "robust" and "complex" groups, respectively. Numbers on main branches show the Bayesian posterior probability and bootstrap support obtained under Maximum Parsimony and Maximum Likelihood criteria, respectively. Asterisks indicate other well-supported clades (pp≥ 95; bootstrap> 70)

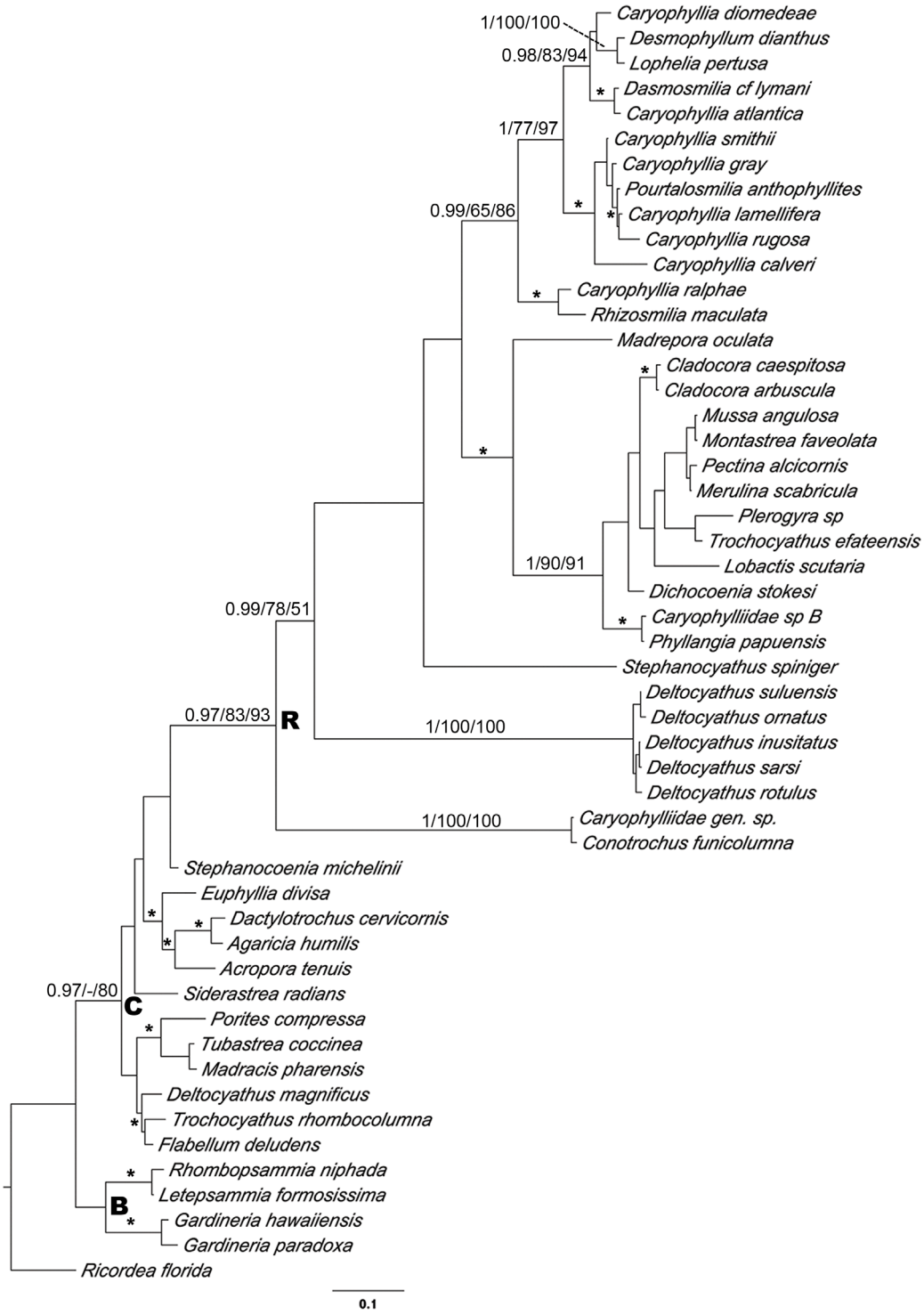

Figure 1.3. Relationship between *Desmophyllum dianthus* and principal taxa from scleractinian families based on mitochondrial COI. Phylogenetic relationships among *D. dianthus* and representative species of the family Caryophylliidae. R, C and B indicate "robust", "complex" and "basal" groups, respectively. The phylogenetic relationships were inferred by BI, MP and ML criteria (numbers show the Bayesian posterior probability and bootstrap supports given at branches, respectively). Asterisks indicate other well-supported clades (pp ≥95; bootstrap > 70).

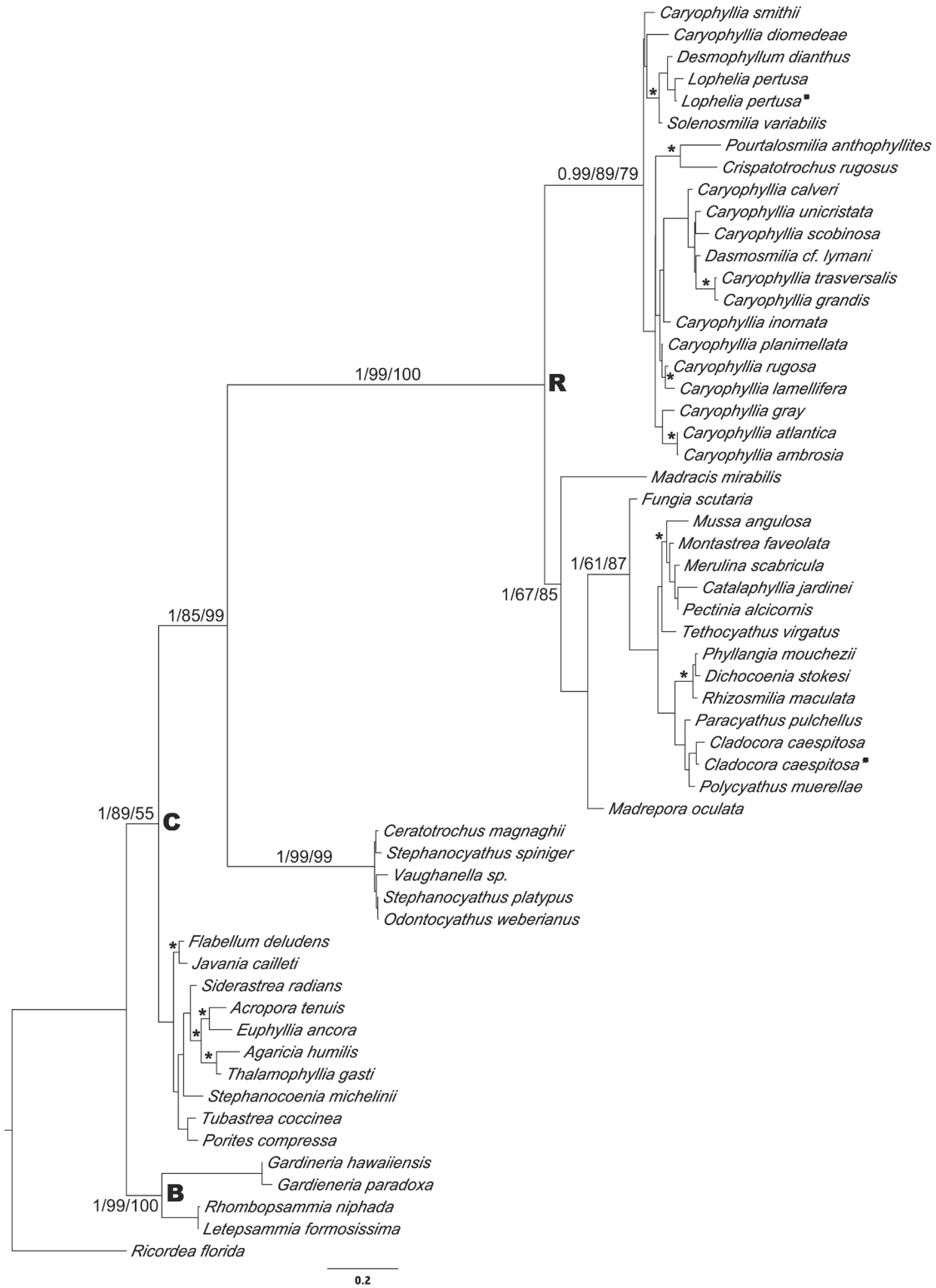

Figure 1.4. Relationship between *Desmophyllum dianthus* and principal taxa from scleractinian families based on mitochondrial 16S rRNA. Phylogenetic relationships among *D. dianthus* and representative species of the family Caryophylliidae. R, C and B indicate "robust", "complex" and "basal" groups, respectively. The phylogenetic relationships were inferred by BI, MP and ML criteria (numbers show the Bayesian posterior probability and bootstrap supports given at branches, respectively). Asterisks indicate other well-supported clades (pp ≥95; bootstrap > 70).

Haplotype network

Relationships within *D. dianthus* haplotypes were represented as a network (Fig. 1.5). Haplotype diversity (Hd) and nucleotide diversity (p) values were highest for COI sequences (0.86 and 0.0052, respectively); however, the ITS alignment was selected for the network analysis as more information existed in the literature for other populations. The ITS network analysis (Fig. 1.5) was conducted considering gaps as missing positions. ITS sequences differed by a maximum of 12 substitutions.

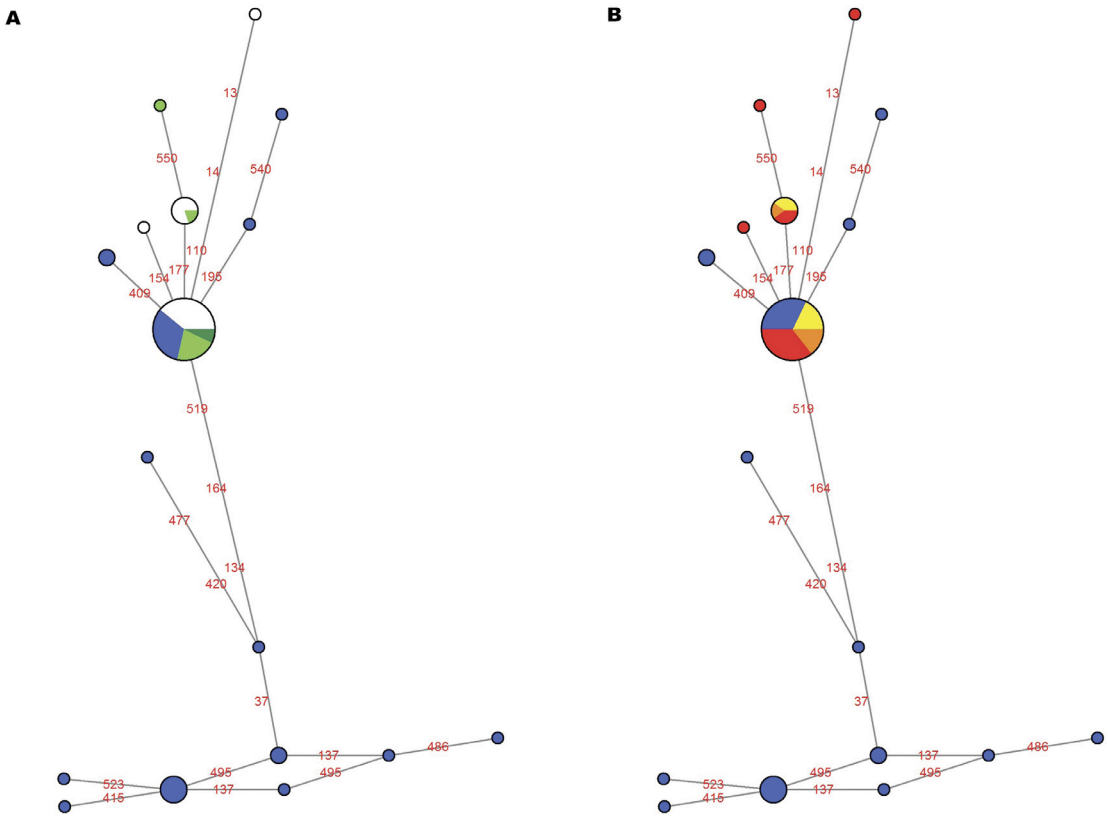

Figure 1.5. Haplotypes network. Parsimony network of internal transcribed spacer (ITS) ribosomal DNA sequence haplotypes of *Desmophyllum dianthus* belonging to Mediterranean Sea populations (from this study) and South Pacific Ocean populations (in blue; from Miller *et al.* 2010-2011). Sizes of the circles are proportional to the number of samples presenting such haplotype. Numbers indicate the variable positions. A) Network based on depth (white= shallow < 600 m; light green= medium 600-1,000 m; dark green= deep> 1000 m). B) Network based on sampling area (red=Ionian Sea; orange=Adriatic Sea; yellow=Strait of Sicily).

Sequences from Mediterranean and South Pacific specimens shared haplotypes and showed the same degree of difference as seen among sequences from Mediterranean samples (Fst= 0.37, p= 0.00). This apparent differentiation did not correspond to any clear geographic structure among populations distributed in regions separated by several thousands of kilometers. Geographic structure was also not found in 16S (Fst= 0.21, p= 0.00; Fig. 1.6), 28S or COI alignments (data not shown).

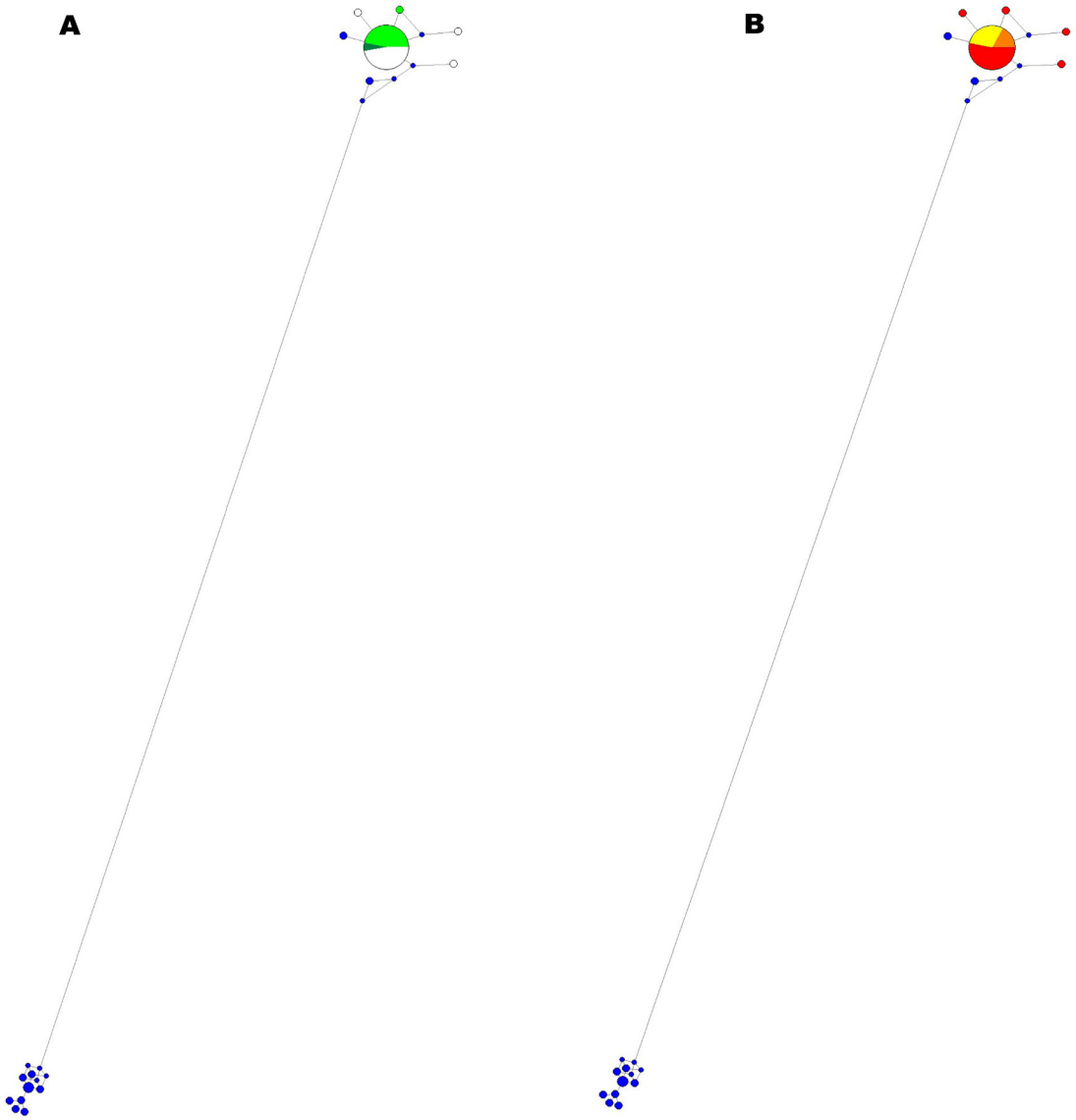

Figure 1.6. Haplotypes network. Parsimony network of mitochondrial 16S rDNA sequence haplotypes of *Desmophyllum dianthus* belonging to Mediterranean Sea populations (from this study) and South Pacific Ocean populations (in blue; from Miller *et al.* 2010-2011). Sizes of the circles are proportional to the number of samples presenting such haplotype. Numbers indicate the variable positions. A) Network based on depth (white= shallow < 600 m; light green= medium 600-1,000 m; dark green= deep> 1000 m). B) Network based on sampling area (red=Ionian Sea; orange=Adriatic Sea; yellow=Strait of Sicily).

Homologous nuclear and mitochondrial DNA sequences were utilized to perform neutrality tests to examine the presence of the evolutionary forces/processes occurring on Mediterranean corals from several locations. Negative Tajima's D and Fu's Fs values (Table 1.3) showed an excess of low frequency alleles and recent mutations, indicating that the Mediterranean population has been evolving randomly and is undergoing population size expansion and/or purifying selection.

Table 1.3. Measures of DNA polymorphism and neutrality tests (Tajima's D and Fu's Fs tests) were performed for nuclear and mitochondrial genes of *Desmophyllum dianthus* specimens. S= segregating (polymorphic) sites; η= total number of mutations; Hd= haplotype diversity; p= nucleotide diversity; D= Tajima's D value; Fs= Fu's Fs value; p= p value.

	Sequences	Sites	S	η	Haplotypes	Hd	p	D	p	Fs	p
ITS	83	643	11	11	7	0,14	0,0006	-2,27	0	-5,78	0,003
28S	79	758	9	9	8	0,17	0,0003	-2,26	0,01	-9,91	0
16S	35	279	16	17	4	0,17	0,0039	-2,45	0,01	0,74	0,245
COI	29	532	21	22	18	0,86	0,0052	-1,84	0,05	-11,21	0

Discussion

Phylogenetic analyses

The three phylogenetic methodologies (ML, MP and BI), based on data from four molecular markers, all supported *D. dianthus* as clearly belonging to the "robust" clade, as previously implied by morphological analyses and molecular analyses of the nuclear 28S rDNA (Cuif *et al.* 2003; Kerr 2005; Stolarski *et al.* 2011) and mitochondrial 16S rDNA and 12S rDNA sequences (Barbeitos *et al.* 2010). Surprisingly, in this study, no clear differentiation between *D. dianthus* and *Lophelia pertusa* was found, in contrast to results from previous molecular and morphological supertree analyses (Kerr 2005). Recently, Huang (Huang 2012) grouped *D. dianthus*, *L. pertusa* and the genus *Caryophyllia* within an unresolved phylogenetic clade. In our molecular phylogeny, *L. pertusa* and *D. dianthus* not only grouped together in a resolved cluster, but also shared haplotypes for some DNA markers (e.g. ITS and COI). This fact illustrates a previously mentioned problem related to the selection of markers analyzed in corals thus far: mitochondrial DNA (i.e. 16S and COI) is less variable in Anthozoans compared to non-Anthozoans (Shearer *et al.* 2002; Hellberg 2006; Chen *et al.* 2009; Huang 2012), and

nuclear markers (i.e. ITS and 28S) often do not provide enough information for coral phylogenetic studies (Chen *et al.* 2004; Vollmer and Palumbi 2004; Coleman and van Oppen 2008; Huang *et al.* 2008; Dueñas and Sánchez 2009). However, recent genome and transcriptome sequencing studies have shown that coral nuclear genomes have a high number of Single Nucleotide Polymorphisms (SNPs), which may represent promising future molecular markers (Meyer and Paulay 2005; Wang *et al.* 2009).

Furthermore, our results support the hypothesis that the morphological similarity of *D. dianthus* and *L. pertusa* reflects a close evolutionary relationship, and provide a new avenue of investigation to study the evolution of acquisition/loss of colonial/solitary life forms between closely related species (Lindner *et al.* 2008; Barbeitos *et al.* 2010; Stolarski *et al.* 2011). The sister species of the *D. dianthus* + *L. pertusa* cluster in the combined analysis was *Caryophyllia calveri*. These are the first molecular data reported for *C. calveri*, showing that it belongs to the "robust" clade (Fig. 1.2) and confirming its placement within *Caryophyllia* (Fig. 1.3 and Fig. 1.4). However, as also envisaged by Kitahara *et al.* (2010a), the supposed monophyly of this genus is disrupted by the inclusion of additional species from the same genus. Herein, our results confirm that *Caryophyllia* does not constitute a monophyletic group due to the presence of *Desmophyllum*, *Lophelia*, *Pourtalosmilia*, *Dasmosmilia*, *Solenosmilia, Crispatotrochus* and *Rhizosmilia* in the same clade. Numerous scleractinian molecular studies have highlighted the lack of agreement between taxonomic classification based on traditional morphological characters and evolutionary lineages recovered by molecular markers (Fukami *et al.* 2008; Stefani *et al.* 2008; Kitahara *et al.* 2010a; Kitahara *et al.* 2010b), prompting a call for an in-depth taxonomic reassessment of the scleractinian genera.

In this regard, the family Caryophylliidae represents a model case. Molecular analyses in which an adequate number of taxa were included support the splitting of this family into multiple different groups (Romano and Cairns 2000). Le Goff Vitry *et al* (2004b). sequenced mitochondrial 16S from 13 taxa identified as Caryophylliidae and found five distinct groups. Currently, one of these clades is now considered as belonging to the family Euphyllidae, but at least four clades of polyphyletic Caryophylliidae remain. Kerr (2005) combined existing molecular and morphological phylogenies into a supertree summary, analyzed 61 taxa of caryophilliid species, and also recovered five clades of Caryophylliidae. Kitahara *et al.* (2010a) analyzed COI in 23 Caryophylliidae

taxa and showed that the family is in nine lineages spread throughout the scleractinian phylogeny. This was also supported by a recent study by Huang (2012).

In the present study, we analyzed data from four different molecular markers and also recovered the polyphyly of Caryophylliidae, consistent with previous studies. However, our results also showed a well-supported differentiation between the *D. dianthus* + *L. pertusa* clade and the *Caryophyllia* genus.

Two possible explanations related to the lack of phylogenetic signal from morphological diagnostic features in corals could account for our observations. One possibility is that the morphological characters used to date are homoplasic, while the other possibility is that the substitution rates of the genes used in this study cannot disentangle the evolutionary history of Scleractinia.

Current strategies for conducting large phylogenetic analyses focus on data combination using supertree and supermatrix methods. Despite their utility, there has been much debate about the relative merits of the best strategy when using sequence data from multiple genes or the utilization of evidence from different datasets, especially when some genes or characters have yet described for some species (Ren *et al.* 2009; Bininda-Emonds 2010).

When such data are missing, both supertree and supermatrix strategies can lack statistical support and ignore uncertainties in the subtrees/matrices (Ren *et al.* 2009; von Haeseler 2012), which sometimes can lead to a misinterpretation of the phylogenetic relationship among species. In fact, the lack of data due to the incomplete presence of genes for all of the species analyzed can yield irregular matrices. This could lead to inferring an erroneous phylogeny; for example, the surprise grouping of *D. dianthus* + *L. pertusa* into a clade, and the clear relationship between this clade and *C. calveri* and *C. smithii*, might have not been observed. Further studies that improve current methodologies or find alternative approaches are necessary to resolve "irresolvable" evolutionary questions.

Haplotype network

Previous studies (Rodriguez-Lanetty and Hoegh-Guldberg 2002; Le Goff-Vitry *et al.* 2004a; Zardus *et al.* 2006; Combosch *et al.* 2008; Eytan *et al.* 2009; Costantini *et al.*

2010; Miller *et al.* 2010; Miller *et al.* 2011) have shown that the nuclear ITS region is more informative for distinguishing between geographically and bathymetrically isolated populations than either of the mitochondrial DNA regions. Patterns have been found within fjords and open slope regions for other deep-sea corals, such as for *L. pertusa* in the northeast Atlantic Ocean (Faure *et al.* 2009), suggesting that gene flow among geographically separated populations may be high. Costantini *et al.* (2010) and Eytan *et al.* (2009) reported depth as a potentially important physical factor and as an isolating mechanism for eurybathic species, which has also been demonstrated in other studies (Rogers 2000; Guinotte *et al.* 2006; Raupach *et al.* 2007; Brandão *et al.* 2010). Other interesting results have been found by Miller *et al.* (2010), where genetic differentiation among seamounts off Tasmania, Australia and the Auckland Islands was apparent only in the coral *D. dianthus* and not in other scleractinians or antipatharians. Nevertheless, in spite of the significant values found by Miller *et al.* (2010) for certain genetic subdivision indices, the analyzed localities still share some common haplotypes. Miller *et al.* (2011) provided evidence of statistically significant levels of genetic differentiation consistent with limited gene flow and isolation, and indicated that depth was a major component of such differentiation. The strongest pattern of depth stratification was found from ITS sequence data. The dynamics of the fluctuation of the oxygen-minimum zone (OMZ) and the shoaling of the aragonite saturation horizon (ASH) may act as barriers to gene flow in the deep sea, leading to speciation in marine invertebrates (Millot and Taupier-Letage 2005; Cairns 2007; Guinotte and Fabry 2008; Reithdorf 2008). Such results can explain how geographically isolated populations from southeast Australia, New Zealand and Chile are genetically subdivided more by their stratigraphic bathymetry than by their geographic distances.

As previously mentioned, our samples were obtained from the Adriatic Sea (276-720 m), Ionian Sea (482-1,102 m) and Strait of Sicily (403-850 m). The intermediate and deep water currents mainly characterizing the area are the Levantine Intermediate Water (LIW), Adriatic Deep Water (AdDW) and Aegean Deep Water currents (AeDW) (Fig. 1.1). The LIW circulates at approximately 200-600 m along the northeastern slope of the Ionian Sea, penetrating into the southern Adriatic Sea, and then continues along a slope as far as the Strait of Sicily, where most of it outflows into the Western Basin (at 400 m). The other two currents first accumulate in the troughs (1,000-1,500 m) over which they are formed (in the southern Adriatic and southern Aegean Seas,

respectively) before outflowing through various openings (Fig. 1.1, Table 1.1) (Rodriguez-Lanetty and Hoegh-Guldberg 2002). These water masses are depth-stratified and may represent two distinct bathymetric levels that could create depth structuring in species diversity and community composition.

Along more than 1,000 km of the southeastern and southwestern Italian slopes in the Mediterranean Sea, most of the specimens analyzed shared common nuclear (i.e. ITS and 28S) and mitochondrial (i.e. 16S and COI) genotypes and/or haplotypes, suggesting high regional connectivity among deep-sea populations from the Adriatic Sea, Ionian Sea and Strait of Sicily.

The well recognized slow evolutionary rate of mitochondrial DNA in corals, estimated to be up to 10 times slower than relative nuclear DNA markers (Shearer *et al.* 2002; Hellberg 2006), may be responsible for the presence of limited numbers of mtDNA haplotypes across all populations. Surprisingly, nuclear data sequences provide no genetic differentiation among Mediterranean populations.

Physical connectivity of Mediterranean sites may be attributed to the principal currents in intermediate and deep waters (Rodriguez-Lanetty and Hoegh-Guldberg 2002; Combosch *et al.* 2008). Moreover, planktonic *D. dianthus* larvae are thought to be retained within natal deep-water masses (Miller *et al.* 2011), and taken together with the patterns of LIW, AdDW and AeDW currents flowing in these regions. We hypothesized that larvae should easily be able to disperse within the region along the southern Italian continental margin, thereby maintaining genetic connectivity among contiguous regions.

Instead, indices of genetic differentiation (F_{ST}= 0.37, p= 0.00) were found among sampling sites distributed in regions separated by supposedly clear geographic barriers, such as the Mediterranean Sea and South Pacific Ocean. Surprisingly, the network analyses also showed haplotypes being shared between these two areas. The occurrence of shared haplotypes between specimens from northern and southern hemispheres could indicate historical patterns of genetic diversity (current or recent gene flow, incomplete lineage sorting or retention of ancestral polymorphism), methodological bias (using genes or regions with a substitution rate inadequate to show divergence) or both (differences in the coalescence of these genes combined with populations divergence).

The extent of gene flow is correlated with reproductive traits, and understanding the processes that limit or promote dispersal in coral species can provide insights into how populations persist and evolve (Nunes *et al.* 2011). Although studies on deep-water coral reproduction are increasing, many life history aspects associated with coral reproductive strategies such as larval longevity, long-distance dispersal potential and mortality, among others, are still poorly understood. Even if it is not unusual for scleractinian species to have differing reproductive patterns (Fadlallah 1983; Harrison 2011) we hypothesize that *D. dianthus* may have similar reproductive strategies as observed in other deep-water corals species (Waller *et al.* 2005): broadcast spawners with lecithotrophic larvae. Since coral larvae are relatively poor swimmers, their dispersal distance largely depends on their longevity (i.e. maximum lifespan), settlement competence, larval survival and oceanographic factors (e.g. speed and direction of water currents, or ocean floor topography) (Scheltema 1986; Pechenik 1990). Graham *et al.* (2008) showed that larval longevities are much greater than previously reported (on the order of 200 days or more), and thus, should be sufficient to allow very long-distance dispersal. Moreover, this study also provided strong support for high early and late mortality of coral larvae, suggesting that although the potential for rare long-distance dispersal events exists, most larvae do not survive long enough to be transported very far (Graham *et al.* 2008). Thus, the majority of successful recruitments are likely to involve settlement on natal or neighboring reefs, particularly given that most larvae become competent to settle quickly, within a few days after spawning (Graham *et al.* 2008). In fact, a recent study on planktonic larval durations (PLDs) by (Burgess and Marshall 2011) suggested that extended PLDs could affect the dynamics of adult populations directly (via reductions in settlement density) and indirectly (via reductions in the post-settlement performance of individuals that experienced a metamorphic delay before settling). Considering the results of the above studies, current or recent gene flow is unlikely given the paleogeographic history of the areas involved in the present study. Some connections can be argued among the different South Pacific populations, even if the distances between them are considerable, but the Mediterranean Sea has been effectively a semi-closed sea since more than five million years ago (Krijgsman *et al.* 1999). Connections with southern Pacific populations are doubtful because of the great distance and possible oceanographic barriers between these biogeographic regions. As demonstrated by

Nunes *et al.* (2011), the combined effects of distance and physical oceanography are likely important isolating factors for coral populations. These barriers may be more permeable for other organisms whose ecologies and life histories permit dispersal at greater distances than those of corals (Nunes *et al.* 2011). Even after selecting the most variable DNA markers (ITS and COI), the hypothesis of continued gene flow among populations cannot be fully supported because differences of haplotype frequencies among populations exist. The shared haplotypes may be ancestral haplotypes that have been maintained over time in the two populations without continued gene flow (Nunes *et al.* 2011). Since reproduction patterns and larval life strategies (e.g. high early mortality rates), and consequently population dynamics, can differ substantially among species (Waller *et al.* 2005; Graham *et al.* 2008), together with the challenging (from an investigative point of view) habitat of CWCs, future studies using a wide variety of approaches (e.g. from molecular genetic to biophysical modeling) have to be developed to extensively study the evolutionary history of these deep-water organisms.

In light of the results obtained in this study, it is apparent that *D. dianthus* and *L. pertusa*, and by extension Caryophylliidae and Scleractinia, need further taxonomic revision due to the lack of taxonomic congruence with observed evolutionary relationships. Therefore, in-depth analyses of new morphological features and molecular markers are critically needed.

None of the nuclear and mitochondrial DNA regions utilized here were useful as exhaustive markers for population studies on *D. dianthus,* and therefore, we could not exclude the hypothesis that the three Mediterranean areas investigated in this study constitute a unique *D. dianthus* population. Furthermore, we could not confirm any genetic structure between populations in the northern and southern hemispheres.

If depth or water circulation are important factors driving isolation, finding adequate molecular markers and morphological characters that can truly show a lack of connectivity between populations at different depths and from different oceans with high statistical significance is an absolute priority. Markers with higher evolutionary rates may be more informative for resolving genetic relationships at different spatial scales and for providing information that reflects current gene flow patterns.

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CHAPTER II

Chapter adapted from:

Addamo AM, Vertino A, Martínez-Baraldés I, López-González PJ, Taviani M, Machordom A (In prep.) Morphological polymorphism throughout a wide ecological and biogeographic range: stability in deep habitats?

Desmophyllum crista galli. Charles Joseph Gravier (1920). Madreporaires provenant des Campagnes des yachts Princesse-Alice et Hirondelle II (1893-1913).

Morphological polymorphism throughout a wide ecological and biogeographic range: stability in deep habitats?

Abstract

Multilevel variations are recognized in hermatypic corals, however high degree of plasticity is also well documented for ahermatypic species. *Desmophyllum dianthus*, a widespread solitary coral, exhibits a high degree of morphological variation in its deep-water forms. This study combines three different morphometric techniques: 1) classical linear external morphology, 2) three-dimensional coordinates landmarks, and 3) linear measurements and counts made on cnidocyst features to assess its pattern of morphological variation. Comparative morphological characterization of specimens of *D. dianthus* throughout a wide ecological and biogeographic range did not show a structured pattern of variation. Hypothesis of intraspecific polymorphism is discussed for the incongruence between appearing variability and obtained results.

Keywords: Scleractinia, morphology, 3D landmarks, cnidocysts, phenotypic plasticity, intraspecific variation.

Introduction

Since the first comprehensive studies on coral systematics based on skeletal characters (Milne Edwards and Haime 1857; Vaughan and Wells 1943), the phylogeny of the order Scleractinia has received renewed attention in the last 20 years thanks to the availability of new molecular and morphological techniques (Chen *et al.* 1995; Veron 1995; Romano and Palumbi 1996; Stanley and Fautin 2001; Stolarski and Roniewicz 2001; Kerr 2005; Fukami *et al.* 2008; Barbeitos *et al.* 2010; Kitahara *et al.* 2010; Stolarski *et al.* 2011; Lin *et al.* 2014).

High levels of phenotypic variability and plasticity encrypt species boundaries and complicate the definition of valid taxonomic units (Todd 2008). Coral often do not meet

the criteria of conventional species concepts due to extreme phenotypic plasticity and/or instances of geographical restriction or hybridization along the genealogical history of the species. For these reasons, several taxa were synonymised due to lack of clear diagnostic morphological characters reflecting species boundaries, or due to the lack of obvious transitions among morphotypes within an acceptable morphological species range (Hoeksema 1993; Kitahara and Cairns 2008; Benzoni *et al.* 2012; Luck *et al.* 2013; Schmidt-Roach *et al.* 2014; Terraneo *et al.* 2014). In order to overcome problems caused by hybridization and phenotypic plasticity, traditional description of coral species based on macromorphological structure of the corallite is often combined to "cartesian coordinates landmarks" or usually called 2-dimensional or 3-dimensional methods (e.g. *Favia, Pocillopora* and *Psammocora* in van Oppen *et al.* 2001; Benzoni *et al.* 2010; Budd *et al.* 2012; Kongjandtre *et al.* 2012; Richards *et al.* 2013; Huang *et al.* 2014; Schmidt-Roach *et al.* 2014), where size and shape coordinates are used as potential characters to discriminate morphospecies (Budd *et al.* 1994; Budd and Stolarski 2009).

In addition, alternative species concept as the "unified species concept' (USC) (De Queiroz 2007) has represented an appropriate taxonomic approach (Schmidt-Roach *et al.* 2014). The USC treats separately evolving metapopulation lineage as the only necessary property of species; hence all criteria associated with previously accepted species concepts represent independent components (operational criteria) that are used in synergy to assess lineage separation. The USC shifts emphasis away from the traditional species criteria, encouraging biologists to develop new methods of species delimitation that are not tied to those properties (De Queiroz 2007).

In this renewed scenario, as clearly stated by Budd *et al.* (2010) molecular phylogenetic analyses have remarkably changed the understanding of scleractinian evolution at all levels, making it possible to test a wide variety of hypotheses derived from mophological studies, from the issue of monophyly of the order to the role of hybridization in Scleractinia evolution (Medina *et al.* 2006; Budd *et al.* 2010; Kitahara *et al.* 2010; Stolarski *et al.* 2011; Lin *et al.* 2014).

At the same time, several authors highlighted cnidocyst as 'organic characters' with new taxonomic informative source and their phylogenetics implications (Pires 1997;

Terrón-Sigler and López-González 2005; Fautin 2009; Picciani *et al.* 2011; Martínez-Baraldés *et al.* 2014).

The genus *Desmophyllum* is known since the Cretaceous (Wells, 1956), however skeletal morphotypes indistinguishable from the modern species *D. dianthus* have been recorded since the Early Miocene and are particularly common in NE Atlantic and Mediterranean Pleistocene deposits (Zibrowius 1987; Vertino 2003; Vertino *et al.* 2014). Due to the paucity of modern deep-water specimens and entire fossil ones, taxonomic studies merely based on few macro-morphological characters (such as size, shape and less commonly number of septa) have led past authors to over-split the genus into too many species. Indeed, at least 11 modern species and over 20 fossil ones - mostly established by Seguenza (1864) - have been recently synonymised to the cosmopolitan species *Desmophyllum dianthus* (Cairns 1995).

Where coral populations are potentially isolated, the possibility that allopatric speciation may occur is high and so it may be that isolated populations of *D. dianthus* have diverged or are in the process of speciation (Miller *et al.* 2011). Molecular and morphological studies have provided evidence for the significant subdivision among populations of *D. dianthus* from three geographic regions (SE Australia, New Zealand and Chile) and from different depth strata (shallow< 600 m, mid 1,000-1,500 m, deep> 1,500 m), suggesting that *D. dianthus* populations across the Southern Ocean have a common ancestor but the populations at different depths within the geographic regions are isolated and have begun to diverge from each other (Miller *et al.* 2011). On the contrary, no fully resolved results were obtained by Fillinger and Richter (2013), who did not find any clear pattern of morphological variability in relation to environmental gradients in a Chilean fjord.

One of the greatest challenges in the delineation of potential *Desmophyllum* species through skeletal analysis is the relative paucity of taxonomic features and the high morphological plasticity of its skeleton (Fig. 2.1). However, detailed morphometric analyses of the corallum of *Desmophyllum* specimens have never carried out so far nor any attempt of identifying and measuring 'organic characters', such as cnydocists, has been made.

The main aim of this study was to test the existence of distinct morphological groups within the worldwide spread species *Desmophyllum dianthus* and to identify, if any, relationships between morphological groups and oceanographic distribution. To test whether morphological variation, within the coral skeleton and tissue, had a specific pattern, three different morphometric approaches were used: 1) morphometry of macroscopic skeletal features; 2) analysis of three-dimensional coordinates of skeletal landmarks; 3) linear measurements and counts made of cnidocyst features.

Figure 2.1. Morphological variability of *Desmophyllum dianthus*. A, B, C, E, J, calyx and corallum of specimens from Chile (USNM 106957, USNM 19168.7, USNM 36367.6, USNM 36367.2 and USNM 19168.9, respectively); D, G, calyx and corallum of specimens from New Zealand (USNM 47412.3 and USNM 94068); I, calyx and corallum of specimen from Ecuador (USNM 84814.2); F, K, calyx and corallum of specimens from Japan (USNM 92612.3 and USNM 92612.5); H, calyx and corallum of specimen from New Caledonia (USNM 1153987).

Material and Methods

Material examined

The specimens examined in the present study are listed in Table 2.1, and consist of: 1) material registered at the U. S. National Museum of Natural History; 2) specimens collected during the Mediterranean Sea and Atlantic Ocean cruises M70-1 (2006), MEDCOR (2009) and ECWC (2012), on board the RRVV *Meteor*, *Urania* and *Belgica*, respectively; 3) specimens collected by SCUBA diving from 12 m depth at Jaime Island (Pitipalena Fjord, Chile).

Prior to preparing the samples for coral skeleton examination, soft tissues were extracted and preserved in absolute ethanol. Each remaining corallum was soaked for 48 hours in 50% sodium hypochlorite solution at room temperature to remove all soft parts, rinsed in freshwater and dried for microscope observation. Coral specimens collected for cnidocysts examination were fixed in 4% buffered formalin-seawater, decalcified in a 10% formic acid solution, and later transferred to 70% ethanol.

Specimens of *D. dianthus* were classified per marine provinces, depth zones, and size classes using informations available in literature about different biogeographical marine areas, provinces and depth zones (Kelleher *et al.* 1995; Spalding *et al.* 2007; Watling *et al.* 2013); whereas, size classes were defined based on the average of growth rate of *D. dianthus*: 1 mm $year^{-1}$ (Adkins *et al.* 2004). The objective was to visually examine any morphological differences in *D. dianthus* from the marine provinces, depth zones, and age classes groups.

Several specimens of *D. dianthus* from 13 different provinces and five depth zones were examined for skeletal macromorphology analysis, and hereafter they are identified with their corresponding geographic codes (Tables 2.1, 2.2, 2.3). Three polyps from the Moira Mounds (Ireland), Jaime Island (Chile), Burdwood Bank (Argentina), Cap de Creus Canyon (Spain), and two specimens from off Washington State (USA) were examined for cnidocyst analysis, and hereafter they are identified as PacS/48, PacN/BY12, AtlN/BY4, AtlS/BY10, and Med/BY4 respectively.

Table 2.1. Information of specimens used for analyses. USNM= National Museum Natural History; MNCN=Museo Nacional de Ciencias Naturales; US= Universidad de Sevilla.

Country	Site	Depth	Museum	Specimen	Province	Macromophology Analysis	Samples for CDA (Fig.5)	3D landmark Analysis	Cnidocyst Analysis
Africa	Gambia	1050	USNM	80988	BY4	√		√	
Africa	Maroc	400	USNM	80404.1	BY4	√			
Africa	Maroc	400	USNM	80404.2	BY4	√			
Argentina	BurdwoodBank	293	USNM	47399	BY13	√			
Argentina	Tierra del Fuego	494	USNM	45669	BY10	√			
Argentina-UK	South Georgia Island	686	USNM	47409	BY10	√	√		
Argentina-UK	South Georgia Island	549	USNM	47407.1	BY10	√	√		
Argentina-UK	South Georgia Island	549	USNM	47407.2	BY10	√	√		
Argentina-UK	South Georgia Island	549	USNM	47407.3	BY10	√	√		
Argentina-UK	South Georgia Island	549	USNM	47407.4	BY10	√	√		
Argentina-UK	South Georgia Island	549	USNM	47407.5	BY10	√	√		
Argentina-UK	South Georgia Island	549	USNM	47407.6	BY10	√	√		
Argentina-UK	South Georgia Island	549	USNM	47407.7	BY10	√			
Australia	Bass Strait	unknown	USNM	85319	BY11	√			
Australia	New South Wales	73	USNM	85320	55/56	√		√	
Australia	Tasmania	1150	USNM	85080	BY10	√		√	
Azores		unknown	USNM	48752	BY4	√			
Azores		1415	USNM	84772	BY4	√			
Azores		784	USNM	48751	BY4	√			
Chile	Aysen del Gen. Carlos Ibanez	821	USNM	36545.1	BY8	√	√		
Chile	Aysen del Gen. Carlos Ibanez	821	USNM	36545.2	BY8	√	√		
Chile	Aysen del Gen. Carlos Ibanez	821	USNM	36545.3	BY8	√	√		
Chile	Aysen del Gen. Carlos Ibanez	821	USNM	36545.4	BY8	√	√		
Chile	Aysen del Gen. Carlos Ibanez	821	USNM	36545.5	BY8	√	√		
Chile	Aysen del Gen. Carlos Ibanez	821	USNM	36545.6	BY8	√	√		
Chile	Aysen del Gen. Carlos Ibanez	821	USNM	36367.1	BY8	√	√		
Chile	Aysen del Gen. Carlos Ibanez	821	USNM	36367.2	BY8	√	√		
Chile	Aysen del Gen. Carlos Ibanez	821	USNM	36367.3	BY8	√	√		
Chile	Aysen del Gen. Carlos Ibanez	821	USNM	36367.4	BY8	√	√		
Chile	Aysen del Gen. Carlos Ibanez	821	USNM	36367.5	BY8	√			
Chile	Aysen del Gen. Carlos Ibanez	821	USNM	36367.6	BY8	√			
Chile	Aysen del Gen. Carlos Ibanez	821	USNM	36367.7	BY8	√			
Chile	Caleta Gonzalo	35	USNM	1009657	48	√			
Chile	Lenca	27	USNM	1009654	48	√			
Chile	Magallanes	544	USNM	47400	BY8	√		√	
Chile	Magallanes	636	USNM	36426.1	BY8	√			
Chile	Magallanes	636	USNM	36426.2	BY8	√			
Chile	Magallanes	636	USNM	36426.3	BY8	√			
Chile	Magallanes	636	USNM	36426.4	BY8	√			
Chile	Magallanes	636	USNM	36426.5	BY8	√			
Chile	Magallanes	636	USNM	19168.1	BY8	√		√	
Chile	Magallanes	636	USNM	19168.2	BY8	√		√	
Chile	Magallanes	636	USNM	19168.3	BY8	√			
Chile	Magallanes	636	USNM	19168.4	BY8	√		√	
Chile	Magallanes	636	USNM	19168.5	BY8	√		√	
Chile	Magallanes	636	USNM	19168.6	BY8	√			
Chile	Magallanes	636	USNM	19168.7	BY8	√			
Chile	Magallanes	636	USNM	19168.8	BY8	√			
Chile	Magallanes	636	USNM	19168.9	BY8	√			
Costarica	Cocos Island	628	USNM	84822.1	BY7	√			
Costarica	Cocos Island	628	USNM	84822.2	BY7	√			
Ecuador	Galapagos (Santiago Island)	780	USNM	84819.1	BY7	√			
Ecuador	Galapagos (Santiago Island)	780	USNM	84819.2	BY7	√			
Ecuador	Galapagos (Santiago Island)	780	USNM	84819.3	BY7	√			
Ecuador	Galapagos (Sta Cruz)	460	USNM	84814.1	BY7	√			
Ecuador	Galapagos (Sta Cruz)	460	USNM	84814.2	BY7	√			
Ecuador	Galapagos(Espanola Island)	unknown	USNM	84816.1	BY7	√			
Ecuador	Galapagos(Espanola Island)	unknown	USNM	84816.2	BY7	√			
Ecuador	Galapagos(Espanola Island)	unknown	USNM	84816.3	BY7	√			
Ecuador	Galapagos(Espanola Island)	unknown	USNM	84816.4	BY7	√			
Ecuador	Roca Redonda	806	USNM	84820.1	BY7	√			
Ecuador	Roca Redonda	806	USNM	84820.2	BY7	√			
Ecuador	Roca Redonda	806	USNM	84820.3	BY7	√			
Ecuador	Roca Redonda	806	USNM	84820.4	BY7	√			
Ecuador	Roca Redonda	806	USNM	84820.5	BY7	√			
Ecuador	Roca Redonda	806	USNM	84820.6	BY7	√			
Ecuador	Roca Redonda	806	USNM	84820.7	BY7	√			
Ecuador	Roca Redonda	806	USNM	84820.8	BY7	√			
Ecuador	Roca Redonda	806	USNM	84820.9	BY7	√			
Ecuador	Roca Redonda	806	USNM	84820.10	BY7	√			
Ecuador	Roca Redonda	806	USNM	84820.11	BY7	√			
Ecuador	Roca Redonda	806	USNM	84820.12	BY7	√			
Ecuador	Roca Redonda	806	USNM	84820.13	BY7	√			
Ecuador	Galapagos (Sta Cruz)	717	USNM	19146	BY7	√			
France	Bay of Biscay	1050	USNM	48745.1	BY4	√			

Table 2.1 (continued). Information of specimens used for analyses. USNM= National Museum Natural History; MNCN=Museo Nacional de Ciencias Naturales; US= Universidad de Sevilla.

Country	Site	Depth	Museum	Specimen	Province	Macromophology Analysis	Samples for CDA (Fig.5)	3D landmark Analysis	Cnidocyst Analysis
France	Bay of Biscay	1050	USNM	48745.2	BY4	√			
France	Gulf of Gasconge	1340	USNM	48748.1	BY4	√		√	
France	Gulf of Gasconge	1340	USNM	48748.2	BY4	√			
France	Gulf of Gasconge	1130	USNM	48759	BY4	√			
Ireland	Celtic Sea	1000	USNM	48746	BY4	√			
Ireland	Celtic Sea	1158	USNM	48747	BY4	√			
Ireland	Mer Cettique	1470	USNM	48740.1	BY4	√	√		
Ireland	Mer Cettique	1470	USNM	48740.2	BY4	√	√		
Ireland	Mer Cettique	1470	USNM	48740.3	BY4	√			
Ireland	Mer Cettique	1470	USNM	48740.4	BY4	√	√		
Ireland	Mer Cettique	1470	USNM	48740.5	BY4	√	√		
Ireland	Mer Cettique	1470	USNM	48740.6	BY4	√	√		
Ireland	Mer Cettique	1470	USNM	48740.7	BY4	√	√		
Ireland	Mer Cettique	1470	USNM	48740.8	BY4	√	√		
Ireland	Mer Cettique	1470	USNM	48740.9	BY4	√	√		
Ireland	Mer Cettique	1470	USNM	48740.10	BY4	√	√		
Italy	Gela	850	MNCN	GEL278	BY4	√			
Italy	Gela	850	MNCN	GEL281	BY4	√		√	
Italy	Gela	850	MNCN	GEL275	BY4	√			
Italy	Rocella Ionica	unknown	MNCN	ROC153	BY4	√	√		
Italy	Rocella Ionica	unknown	MNCN	ROC185	BY4	√	√	√	
Italy	Rocella Ionica	unknown	MNCN	ROC186	BY4	√			
Italy	Rocella Ionica	unknown	MNCN	ROC178	BY4	√			
Italy	Rocella Ionica	unknown	MNCN	ROC177	BY4	√	√	√	
Italy	Rocella Ionica	unknown	MNCN	ROC151	BY4	√	√		
Italy	Rocella Ionica	unknown	MNCN	ROC171	BY4	√	√		
Italy	Rocella Ionica	unknown	MNCN	ROC150	BY4	√	√	√	
Italy	Rocella Ionica	unknown	MNCN	ROC149	BY4	√		√	
Italy	Rocella Ionica	unknown	MNCN	ROC170	BY4	√		√	
Italy	Rocella Ionica	unknown	MNCN	ROC165	BY4	√	√		
Italy	Rocella Ionica	unknown	MNCN	ROC146	BY4	√	√		
Italy	Rocella Ionica	unknown	MNCN	ROC174	BY4	√	√		
Italy	Rocella Ionica	unknown	MNCN	ROC175	BY4	√	√		
Italy	Urania Bank		MNCN	URA101	BY4	√			
Japan	Kagoshima	715	USNM	M547422.1	BY12	√	√	√	
Japan	Kagoshima	715	USNM	M547422.2	BY12	√	√		
Japan	Kagoshima	715	USNM	M547422.3	BY12	√	√		
Japan	Sagama Bay	unknown	USNM	92612.1	BY12	√	√		
Japan	Sagama Bay	unknown	USNM	92612.2	BY12	√	√	√	
Japan	Sagama Bay	unknown	USNM	92612.3	BY12	√	√	√	
Japan	Sagama Bay	unknown	USNM	92612.4	BY12	√	√	√	
Japan	Sagama Bay	unknown	USNM	92612.5	BY12	√	√	√	
Japan	Sagama Bay	unknown	USNM	92612.6	BY12	√	√	√	
Malta	Malta	690	MNCN	MAL198	BY4	√			
Malta	Malta	690	MNCN	MAL219	BY4	√			
Malta	Malta	690	MNCN	MAL195	BY4	√		√	
Malta	Malta	690	MNCN	MAL223	BY4	√			
Malta	Malta	690	MNCN	MAL233	BY4	√			
Malta	Malta	690	MNCN	MAL215	BY4	√			
Malta	Malta	691	MNCN	MAL222	BY4	√			
New Caledonia	Coral Sea	400	USNM	1153977	BY12	√			
New Caledonia	Coral Sea	245	USNM	1153987	BY12	√			
New Zealand	Campbell Rise	421	USNM	47413.1	BY10	√			
New Zealand	Campbell Rise	421	USNM	47413.2	BY10	√			
New Zealand	Campbell Rise	421	USNM	47413.3	BY10	√			
New Zealand	Campbell Rise	421	USNM	47413.4	BY10	√			
New Zealand	Campbell Rise	421	USNM	47413.5	BY10	√			
New Zealand	Campbell Rise	421	USNM	47413.6	BY10	√			
New Zealand	Campbell Rise	421	USNM	47413.7	BY10	√			
New Zealand	Campbell Rise	421	USNM	47413.8	BY10	√			
New Zealand	Campbell Rise	421	USNM	47413.9	BY10	√			
New Zealand	Campbell Rise	421	USNM	47413.10	BY10	√			
New Zealand	Campbell Rise	421	USNM	47413.11	BY10	√			
New Zealand	Campbell Rise	421	USNM	47413.12	BY10	√			
New Zealand	Campbell Rise	421	USNM	47413.13	BY10	√			
New Zealand	Campbell Rise	421	USNM	47413.14	BY10	√			
New Zealand	Campbell Rise	421	USNM	47413.15	BY10	√			
New Zealand	Campbell Rise	421	USNM	47413.16	BY10	√			
New Zealand	Macquarie Ridge	372	USNM	47394	BY10	√			
New Zealand	Milford Sound	25	USNM	94068	54	√			
New Zealand	Milford Sound	30	USNM	94072	54	√			
New Zealand	Off Antipodes Island	540	USNM	47412.1	BY10	√		√	
New Zealand	Off Antipodes Island	540	USNM	47412.2	BY11	√		√	
New Zealand	Off Antipodes Island	540	USNM	47412.3	BY12	√		√	
New Zealand	Three Kings Ridge	1059	USNM	94070	BY6	√		√	

Table 2.1 (continued). Information of specimens used for analyses. USNM= National Museum Natural History; MNCN=Museo Nacional de Ciencias Naturales; US= Universidad de Sevilla.

Country	Site	Depth	Museum	Specimen	Province	Macromophology Analysis	Samples for CDA (Fig.5)	3D landmark Analysis	Cnidocyst Analysis
Panama	Gulf of Panama	838	USNM	22074	BY7	√			
USAE	Bahamas	1280	USNM	46485	BY4	√			
USAE	Bermuda	1200	USNM	83000	BY4	√			
USAE	Bermuda	2174	USNM	1025741.1	BY4	√		√	
USAE	Bermuda	2174	USNM	1025741.2	BY4	√			
USAE	Bermuda	609	USNM	99383	BY4	√			
USAE	Florida Keys	582	USNM	80987.1	BY4	√		√	
USAE	Florida Keys	583	USNM	80987.2	BY4	√			
USAE	Gulf of Mexico (Lousiana)	634	USNM	1071863	BY4	√			
USAE	Maryland	790	USNM	62311.1	BY4	√		√	
USAE	Maryland	790	USNM	62311.2	BY4	√			
USAE	New Jersey	1900	USNM	62308	BY4	√			
USAE	Sooth Caroline	2200	USNM	91933	BY4	√			
USAE	Strait of Florida	1021	USNM	46481	BY4	√			
USAE	Strait of Florida	1200	USNM	46483	BY4	√		√	
USAW	California	274	USNM	19249.1	BY3	√	√		
USAW	California	274	USNM	19249.2	BY3	√	√		
USAW	California	274	USNM	19249.3	BY3	√	√		
USAW	California	274	USNM	19249.4	BY3	√	√		
USAW	Gulf of California	1097	USNM	78595	BY7	√			
USAW	Gulf of California	488	USNM	83583.1	BY7	√			
USAW	Gulf of California	489	USNM	83583.2	BY7	√			
USAW	Hawai'i	346	USNM	20730	BY14	√			
USAW	Washington	312	USNM	78630	BY3	√	√	√	
Ireland	Moira Mounds	1069	MNCN	ECWC10BC5-1	BY4				√
Ireland	Moira Mounds	1069	MNCN	ECWC10BC5-2	BY4				√
Ireland	Moira Mounds	1069	MNCN	ECWC10BC5-3	BY4				√
Chile	Isla Jaime-Pitipalena Fjord	15	MNCN	IJC-1	48				√
Chile	Isla Jaime-Pitipalena Fjord	15	MNCN	IJC-2	48				√
Chile	Isla Jaime-Pitipalena Fjord	15	MNCN	IJC-3	48				√
USA	Washington	183-1280	USNM	100837	BY12				√
USA	Washington	183-1280	USNM	100837	BY12				√
Argentina	Burwood Bank	700-800	US		BY10				√
Argentina	Burwood Bank	700-800	US		BY10				√
Argentina	Burwood Bank	700-800	US		BY10				√
Spain	Cap de Creus	100-400	US		BY4				√
Spain	Cap de Creus	100-400	US		BY4				√
Spain	Cap de Creus	100-400	US		BY4				√

Table 2.2. Legend for marine biogeographical provinces.

Symbol	Province	References
48	Magellanic	Spalding et al. 2007
54	Southern New Zealand	Spalding et al. 2007
55/56	East Centralian /Southeast Australian Shelf	Spalding et al. 2007
BY3	North Pacific Boreal	Watling et al. 2013
BY4	North Atlantic Bathyal	Watling et al. 2013
BY6	New Zealand Kermadec	Watling et al. 2013
BY7	Cocos Plate	Watling et al. 2013
BY8	Nazca Plate	Watling et al. 2013
BY10	Subantarctic	Watling et al. 2013
BY11	Indian Ocean Bathyal	Watling et al. 2013
BY12	West Pacific Bathyal	Watling et al. 2013
BY13	South Atlantic	Watling et al. 2013
BY14	North Pacific Bathyal	Watling et al. 2013

Table 2.3. Legend for marine depth zones.

Symbol	Depth	Range depth in dataset (m)
SW	Shallow water zone	25-73
MW	Mesopelagic water zone	245-293
UB	Upper bathyal zone	312-790
LB	Lower bathyal zone	806-2200
NA	Unknown	unknown

Skeletal analysis: morphometry of macrocharacters

Quantitative morphometric approach was used to compare macromorphological skeletal characters of *D. dianthus*. Twenty characters were measured in 174 individuals including: corallum height (H), corallum length (L), corallum diameters (GD1-GD4 and LD1-LD4), angle (α), total number of costae (CxN), number of costal cycle (Cx), costae length (Cc), presence of discontinue costae (Cd), teca thickness (CT), fossa diameters (GFD and LFD), total number of septa (SxN), number of septal cycles (Sx), septa externess (SExV and SEvH), septa width (SW), and septa thickness (ST and SD5T) (Figure 2.2 and Table 2.4). In order to compare data available from literature, a mean of 5 measurements/corallum were made for septa width, septa extension, septa thickness, and costae length (Miller *et al.* 2011). All characters were measured using stereomicroscope with 10× and 25× magnifications and an ocular micrometer, but morphometric character α was measured on digital images using ImageJ64 software (Syed *et al.* 2009). For each specimen digital images of calyx (front) and corallum (side) were taken with a Nikon D5000 camera. Morphological character ratios (C:S; GCD:L; GCD:LCD, and L:α) were also considered in order to determine morphological variation in defined biogeographical provinces.

Statistical description of morphological ratio in *D. dianthus* and biogeographical variability in the 13 provinces were represented by box plots, using ggplot2 implemented in R environment (Wickham 2009; RStudio 2012).

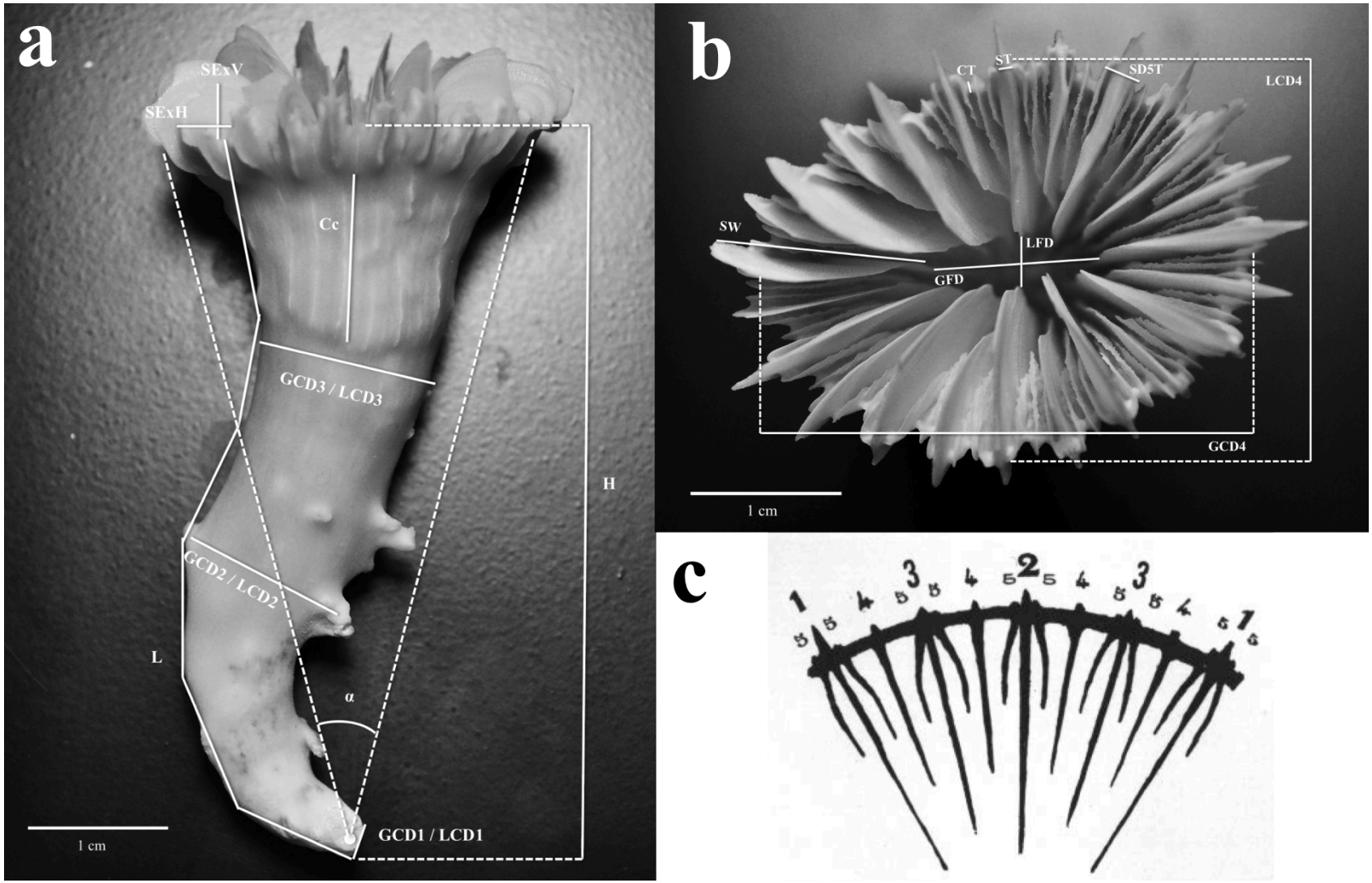


Figure 2.2. Morphometric parameters of corallum (a) and calyx (b) included in the analyses. Diagrammatic representation of septa cycle (c). For abbreviations see Table 2.4.

Table 2.4. Characters included in the macromorphological analysis. *GCD=GCD4.

Symbol	Parameter definition
H	Corallite height
L	Corallite length
GCD4	Greater calicular diameter (costae included)
LCD4	Lower calicular diameter (costae included)
GCD1-3	Greater calicular diameter section 1-3
LCD1-3	Lower calicular diameter section 1-3
PD	Pedicel diameter
α	Angle pedicel-calice
Cx	Costal cycle
CxN	Total costae number
Cc	Costa length
Cd	Presence costae discontinue
CT	Thickenss teca
GFD	Greater Fossa Diameter
LFD	Lower Fossa Diameter
Sx	Septa cycle
SxN	Total septal number
SExV	Vertical exertness of septa
SExH	Horizontal exertness of septa
SW	Max dominant septa width
ST	Dominant septa thickness
SD5T	Dominant septa thickness 5th septa included
C:S	Ratio of number costae to septa
GCD*:L	Ratio of greater calicular diameter to length of corallum
GCD*:LCD	Ratio of greater calicular diameter to lower diameter of corallum
L:α	Ratio length of corallum to angle pedicel-calice

Table 2.5. Legend for size class based on length of corallum. Average growth rate =1 mm/years.

Symbol	Classe	Length range in datase (mm)
1T	1st tridecade	7.9-29.9
2T	2nd tridecade	30-59.9
3T	3rd tridecade	60-89.9
4T	4th tridecade	90-129.9
>5T	over 5th tridecade	>200

Canonical discriminant analysis (CDA) was carried out considering the marine provinces, depth zones, and size classes as the dependent variables (the groups) and the morphometric characters as independent variables (the predictors). The *a priori* marine provinces, depth zones and size classes groups were used to compare morphological characters among individuals (Tables 2.2, 2.3 and 2.5). These analyses were run using SPSS 20.0 (SPSS 2011). The stepwise CDA was used to identify the most influential

morphometric characters in the differentiation of the explored species using the Wilks lambda method. The classification was evaluated by using the Jack-knifed validation.

In order to test for morphological changes during the transition from juvenile to adult form, linear correlation and regression were performed using MASS implemented in R environment (Venables and Ripley 2002; RStudio 2012).

Skeletal analysis: 3D coordinates of landmarks

Three-dimensional morphometric method was chosen for this study, where the entire corallum has been analysed and the data have been easily manipulated and converted to 2D data. Morphometrics analyses were conducted on corallum, the entire skeleton of a solitary coral. Each corallum consists of a tube (corallum wall) with vertical plates that radiate inward and outward (calyx) from corallum wall. A complete vertical plates is termed costosepta (Carlon and Budd 2002b; Budd and Stolarski 2009). A total of 99 coralla were selected based on number of complete septa cycles (> S_4) for morphometrics analysis, but only 32 of them were measured, due to characteristics of skeleton: 18 specimens from the Pacific Ocean, 6 specimens from the Atlantic Ocean, and 8 specimens from the Mediterranean Sea (see Table 2.1). The quality of the specimens had to be evaluated when choosing which ones were to be measured: only relatively complete specimens could be used because of the nature of the landmark methods employed. Juveniles were avoided when possible, as it is possible that shape and proportions may be similar to other scleractinian coral throughout ontogeny. Juvenile condition was established *a priori* as characterized by a corallum length < 15 mm, and septa cycles ≤ S_4.

A combination of type 1 and type 2 landmarks was chosen. Type 1 landmarks are points that can be defined locally, usually an intersection of three structures (Bookstein 1991). The type 1 landmarks in this study consist of junctions between septa and wall that are reliably homologous. In addition, some homologous points that may have a minimal relationship with the shape variation associated with differences in growth rate were also chosen. Type 2 landmarks are defined by a relative local property such as a maximum and minimum of curvature (Bookstein 1991). The type 2 landmarks in this study consist of those that define the length as well as width of septa.

For each corallum a different set of landmarks along costosepta and corallum were digitized using 3D Cartesian coordinates (x–y–z) landmarks (see Budd *et al.*, 1994) for detailed methodology) using a Reflex microscope; several pairs of landmarks endpoints were used to calculate linear distances until the definition of those landmarks able to reflect the shape of costosepta and corallum (Figure 3a-b). Once the landmarks were selected, all the 3D Cartesian coordinates were first used to construct a wireframe (lengths for each pair of endpoints). This was done in IMP-WireMan7. Next Bookstein coordinates calculation and Procrustes superimposition (shape coordinates and centroid size values) were performed using the IMP-Simple3D (Sheets 2004). Variables from 3D landmarks endpoints data matrix were subjected to a principal componets analysis (PCA), which was then performed using the Procustes data with IMP-ThreeDPCA (Sheets 2004). Finally, a canonical variates analysis (CVA) was performed on relative warp scores in IMP-CVAGen6 to investigate variation between the defined group of specimens from different marine provinces.

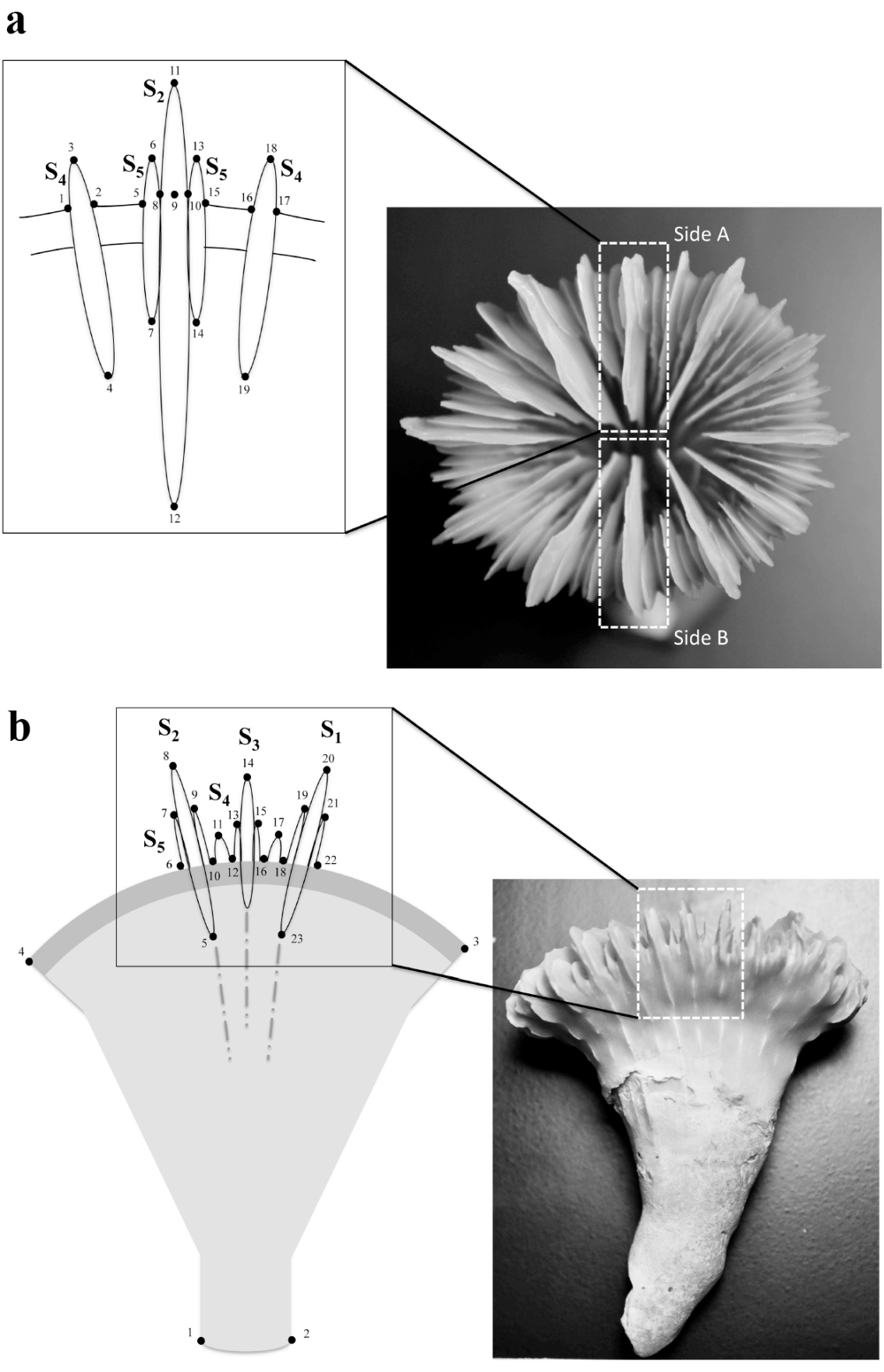


Figure 2.3. Landmarks of calyx (a) and corallum (b) included in the analyses. S= septa cycle.

Tissue analysis: characterization of the cnidom

Squash preparations of tissue from different parts of the polyps (scapus, tentacles, pharynx, and mesenterial filament) were made in order to perform cnidocyst examinations. Observations, measurements and categorizations of undischarged capsules, using LEICA DMLB and ZEISS Axio ScopeA1 Microscopes with Nomarski intereference contrast optics at maximum magnification 100×, were made following the methodology described in Godknecht and Tardent (1989) and Martínez-Baraldés *et al.* (2014), respectively. Two main types of cnidae were analysed: spirocysts (Sp) and nematocysts. Following traditional nomenclature (Weill 1934) and amendments (Carlgren 1940) the observed nematocysts were identified as holotrich (H), basitrich (Bs), and microbasic p-mastigophore (MpM). Each type and categories were recognized according to differences in size range (length, width of capsule and shafts). The different categories were labelled with a consecutive number from smaller to larger size-classes and a letter indicating the tissue where present. For the different tissues the following nomenclature was used; S= scapus, T= tentacle, P= pharynx, and MF= mesenterial filament. For the following cnidae comparison and analyses, coefficient of similarity, classification by hierarchical clustering and by discriminant analysis were performed following usual data analysis procedures (Martínez-Baraldés *et al.* 2014).

Statistical description of cnidom composition in *D. dianthus* and biogeographical variability in the 5 localities were represented by box plots, using ggplot2 implemented in R environment.

Similarity relationships among sampling stations were obtained by the Bray-Curtis index (Bray and Curtis 1957), using the length averages of each cnida in each kind of tissue; additionaly, original data from Terrón-Sigler and López-González (2005) and Martínez-Baraldés *et al.* (2014) were included in order to show the interspecific relationships. Hierarchical agglomerative clustering method was applied using PRIMER v6 program (Clarke and Gorley 2006).

Phylogenetic relationships were explored using the software Winclada-NONA (Goloboff 1999; Nixon 1999-2002), based on the principle of parsimony using the presence-absence matrix obtained for all cnidae in each tissue. For this analysis, the available data from a number of different scleractinian, corallimorpharian and

actiniarian species were also included (see Martínez-Baraldés *et al.* 2014). Corallimorpharia and Actiniaria species were used as an external group. Fitch's parsimony (non-additive) was considered for all characters, since it is unknown if any transformation sequence exists among cnidae categories (Fautin 2009). The consensus clustering was based on a TBR-heuristic analysis, with 100 replicates to test the nodes support.

CDA was carried out considering the localities as the dependent variable (the groups) and the cnidae from each tissue as independent variables (the predictors). The *a priori* five groups used were the same caryophylliid species as mentioned above and previously compared. Only common cnidae were selected for the CDA, except for common cnidae that were present in too low frequencies according to the recommendations of the model. A total of eleven cnidae were included in the analysis (Bs1S, MpM1S, SpT, H1T, Bs2P, Bs3P, MpM1P, Bs1MF, MpM2MF, and MpM4MF). This analysis was run using SPSS 20.0. Its objective was to explore the discriminatory capacity of cnidae while trying to classify new observations. The stepwise CDA was used to identify the most influential cnidae tissue in the differentiation of the explored species using the Wilks lambda method. However, the choice of the common cnidocysts caused differences in the size of the groups, and this was taken into consideration using prior probabilities for each group. The classification was evaluated by using the Jack-knife validation.

Results

Skeletal analysis: morphometry of macrocharacters

Variability and distribution of morphological characters in the 13 provinces studied were compared, but PD and Cd characters were not considered due to large amount of missing data (Table 2.6). All characters were found to be quite constant throughout the marine provinces, except for morphological ratios GCD:L and L:α that showed relevant differences among provinces considered in this study (Figure 2.4).

Table 2.6. Mean (M), standard deviation (SD) and range of each parameter included in the analyses per marine province.

Province	No. Specimen	Statistic	H	L	GD4	LD4	GD1	LD1	GD2	LD2	GD3	LD3	α	Cx
48	2	M ± SD	40,26±25,85	168,25±152,81	28,38±0,78	19,63±1,33	8,62±1,49	7,94±0,28	15,05±0,28	11,08±2,25	19,28±5,66	16,56±3,62	34,09±19,5	2+
		Range	21,98-58,54	60,19-276,3	27,83-28,93	18,69-20,57	7,56-9,67	7,74-8,14	14,85-15,24	9,49-12,67	15,27-23,28	14-19,12	20,29-47,88	
54	2	M ± SD	28,82±8,31	28,82±8,31	31,58±9,83	22,44±3,9	15,11±4,43	12,81±5,08	16,25±5,99	13,66±4,04	21,46±6,04	15,99±0,9	92,18±7,57	3+
		Range	22,94-34,69	22,94-34,69	24,63-38,53	19,68-25,2	11,97-18,24	9,22-16,4	12,01-20,48	10,8-16,52	17,19-25,73	15,35-16,62	86,83-97,53	
BY10	30	M ± SD	46,74±22,17	56,81±22,45	34,53±14,13	24,89±9,22	7,41±3,54	6,46±2,59	12,18±4,39	10,19±2,88	18,84±7,51	14,36±5,04	44,51±11,93	3+
		Range	13,37-106,43	28,08-124,68	9,53-63,53	8,99-42,29	2,24-17,93	2,23-12,82	5-22,02	5,28-15,79	6,9-34,53	5,93-24,24	18,97-72,04	
BY12	11	M ± SD	36,62±12,25	39,55±14,82	29,52±11,82	21,85±7,64	7,87±3,13	8,16±2,65	10,48±4,01	9,06±3,3	16,07±6,53	11,59±4,27	62,78±23,75	2+
		Range	14-51,6	14-60,34	10,32-47,92	9,82-31,52	3,23-13,44	3,38-12,44	4,06-16,99	4,07-14,02	7,5-30,11	5,48-18,82	29,63-114,28	
BY3	5	M ± SD	37,09±11,5	40,69±10,83	29,42±14,42	20,37±5,44	8,89±5,35	8,17±4,44	12,9±3,21	10,93±2,18	16,75±5,3	13,34±2,78	63,32±41,95	2+
		Range	28,57-56,36	29,73-58,24	16,24-46,58	15,18-26,75	3,17-17,18	3,02-14,8	9,55-17,8	8,64-14,49	10,56-24,19	9,44-16,7	23,26-132,88	
BY4	62	M ± SD	36,64±32,62	30,92±13,23	19,98±9,04	16,24±6,59	6,17±2,98	5,68±2,41	9,38±4,68	8,04±3,23	13,55±6,78	11,32±4,58	44,4±16,41	3+
		Range	7,2-255	7,99-68,6	4,11-47,8	3,17-37,35	1,75-18,03	1,18-14,87	2,54-28,42	2,19-18,66	1,75-37,24	2,78-27,78	6,54-76,89	
BY7	29	M ± SD	32,17±15,88	32,2±14,81	22,48±15,11	17,44±10,31	6,35±3,4	6,24±3,84	8,3±3,8	7,51±3,38	11,55±5,16	9,93±4,41	45,82±21,34	3+
		Range	7,63-70,99	9,66-70,63	5,23-64,56	4,38-45,14	2,18-17,9	2,48-21,06	2,92-19,59	2,94-19,1	4,27-26,87	3,93-23,05	16,42-108,4	
BY8	28	M ± SD	49,71±31,39	59,53±32,05	37,06±23,88	29,08±16,83	8,64±5,43	8,97±5,87	12,38±8,11	10,3±5,12	18,89±11,64	14,34±7,35	45,48±17,93	2+
		Range	10,46-128,24	9,16-128,24	3,08-85,97	7,93-68,41	0,67-21,46	3,16-23,78	3,5-39,4	3,46-18,42	5,32-47,24	4,85-29,9	19,56-89,15	
BY11	1	VALUE	83,95	83,95	42,11	30,03	7,07	7,41	11,06	10,62	23,21	15,68	59,32	3
BY13	1	VALUE	62,74	98,53	35,4	27,26	10,88	10,35	17,89	16,61	31,83	22,8	45,1	2+
BY14	1	VALUE	29,17	29,17	25,29	23,69	7,62	7,39	13,04	10,65	22,92	20,32	66,65	3
BY6	1	VALUE	16,2	16,95	13	10,79	3,67	3,53	4,47	4,44	8,28	7,25	52,91	3
55/66	1	VALUE	45,87	45,87	44,34	34,26	17,95	17,11	23,77	21,12	31,7	22,98	80,13	-

Table 2.6 (continued). Mean (M), standard deviation (SD) and range of each parameter included in the analyses per marine province.

Province	No. Specimen	Statistic	H	L	GD4	LD4	GD1	LD1	GD2	LD2	GD3	LD3	α	Cx
48	2	M ± SD	40,26±25,85	168,25±152,81	28,38±0,78	19,63±1,33	8,62±1,49	7,94±0,28	15,05±0,28	11,08±2,25	19,28±5,66	16,56±3,62	34,09±19,5	2+
		Range	21,98-58,54	60,19-276,3	27,83-28,93	18,69-20,57	7,56-9,67	7,74-8,14	14,85-15,24	9,49-12,67	15,27-23,28	14-19,12	20,29-47,88	
54	2	M ± SD	28,82±8,31	28,82±8,31	31,58±9,83	22,44±3,9	15,11±4,43	12,81±5,08	16,25±5,99	13,66±4,04	21,46±6,04	15,99±0,9	92,18±7,57	3+
		Range	22,94-34,69	22,94-34,69	24,63-38,53	19,68-25,2	11,97-18,24	9,22-16,4	12,01-20,48	10,8-16,52	17,19-25,73	15,35-16,62	86,83-97,53	
BY10	30	M ± SD	46,74±22,17	56,81±22,45	34,53±14,13	24,89±9,22	7,41±3,54	6,46±2,59	12,18±4,39	10,19±2,88	18,84±7,51	14,36±5,04	44,51±11,93	3+
		Range	13,37-106,43	28,08-124,68	9,53-63,53	8,99-42,29	2,24-17,93	2,23-12,82	5-22,02	5,28-15,79	6,9-34,53	5,93-24,24	18,97-72,04	
BY12	11	M ± SD	36,62±12,25	39,55±14,82	29,52±11,82	21,85±7,64	7,87±3,13	8,16±2,65	10,48±4,01	9,06±3,3	16,07±6,53	11,59±4,27	62,78±23,75	2+
		Range	14-51,6	14-60,34	10,32-47,92	9,82-31,52	3,23-13,44	3,38-12,44	4,06-16,99	4,07-14,02	7,5-30,11	5,48-18,82	29,63-114,28	
BY3	5	M ± SD	37,09±11,5	40,69±10,83	29,42±14,42	20,37±5,44	8,89±5,35	8,17±4,44	12,9±3,21	10,93±2,18	16,75±5,3	13,34±2,78	63,32±41,95	2+
		Range	28,57-56,36	29,73-58,24	16,24-46,58	15,18-26,75	3,17-17,18	3,02-14,8	9,55-17,8	8,64-14,49	10,56-24,19	9,44-16,7	23,26-132,88	
BY4	62	M ± SD	36,64±32,62	30,92±13,23	19,98±9,04	16,24±6,59	6,17±2,98	5,68±2,41	9,38±4,68	8,04±3,23	13,55±6,78	11,32±4,58	44,4±16,41	3+
		Range	7,2-255	7,99-68,6	4,11-47,8	3,17-37,35	1,75-18,03	1,18-14,87	2,54-28,42	2,19-18,66	1,75-37,24	2,78-27,78	6,54-76,89	
BY7	29	M ± SD	32,17±15,88	32,2±14,81	22,48±15,11	17,44±10,31	6,35±3,4	6,24±3,84	8,3±3,8	7,51±3,38	11,55±5,16	9,93±4,41	45,82±21,34	3+
		Range	7,63-70,99	9,66-70,63	5,23-64,56	4,38-45,14	2,18-17,9	2,48-21,06	2,92-19,59	2,94-19,1	4,27-26,87	3,93-23,05	16,42-108,4	
BY8	28	M ± SD	49,71±31,39	59,53±32,05	37,06±23,88	29,08±16,83	8,64±5,43	8,97±5,87	12,38±8,11	10,3±5,12	18,89±11,64	14,34±7,35	45,48±17,93	2+
		Range	10,46-128,24	9,16-128,24	3,08-85,97	7,93-68,41	0,67-21,46	3,16-23,78	3,5-39,4	3,46-18,42	5,32-47,24	4,85-29,9	19,56-89,15	
BY11	1	VALUE	83,95	83,95	42,11	30,03	7,07	7,41	11,06	10,62	23,21	15,68	59,32	3
BY13	1	VALUE	62,74	98,53	35,4	27,26	10,88	10,35	17,89	16,61	31,83	22,8	45,1	2+
BY14	1	VALUE	29,17	29,17	25,29	23,69	7,62	7,39	13,04	10,65	22,92	20,32	66,65	3
BY6	1	VALUE	16,2	16,95	13	10,79	3,67	3,53	4,47	4,44	8,28	7,25	52,91	3
55/66	1	VALUE	45,87	45,87	44,34	34,26	17,95	17,11	23,77	21,12	31,7	22,98	80,13	-

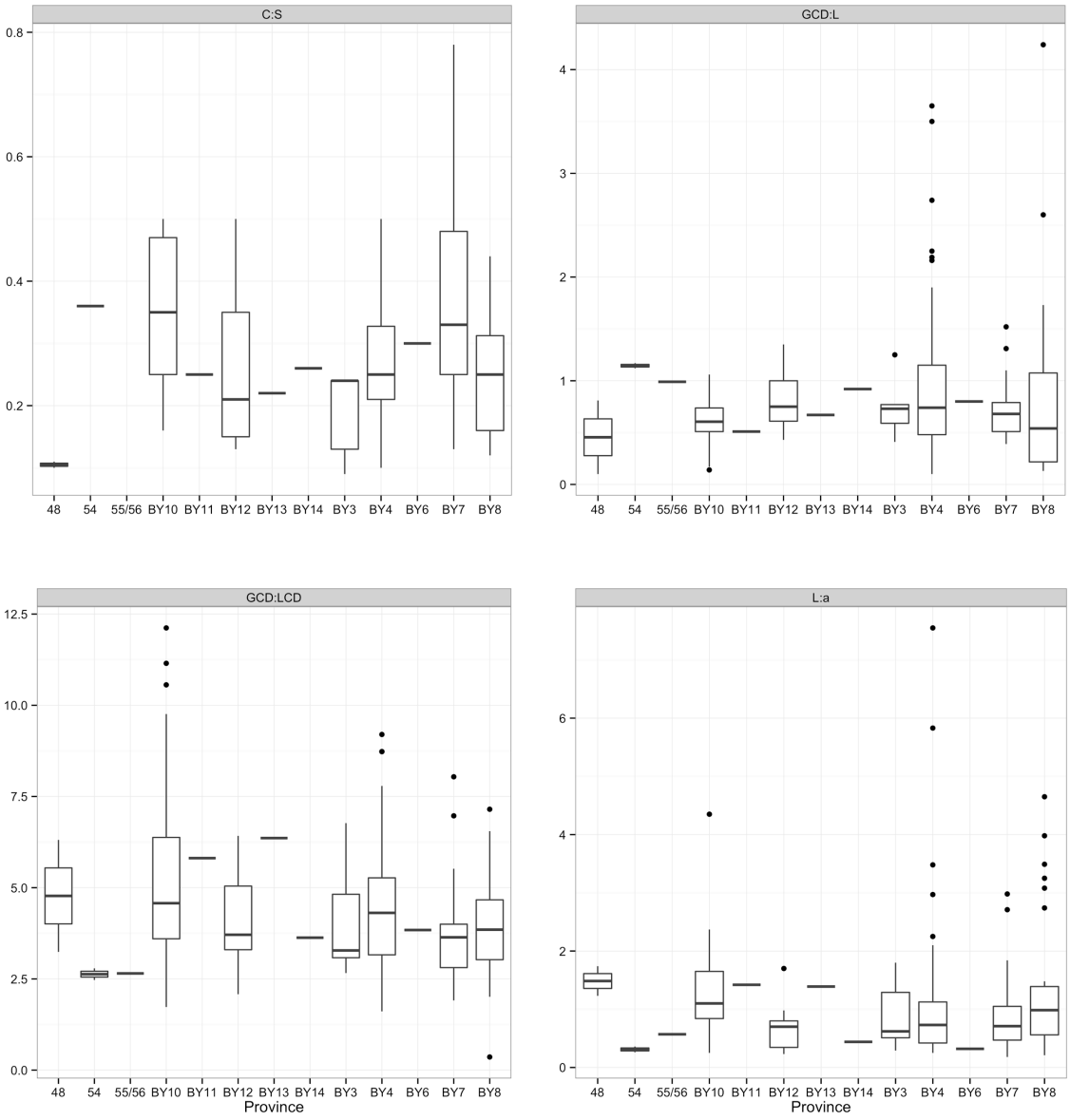


Figure 2.4. Box plots of ratio of morphological characters throughout marine province.

CDA was performed on a total of 50 individuals from the same five marine provinces that those used in the cnidocyst analysis: PacN/BY12, PacS/BY8, AtlN/BY4, AtlS/BY10, and Med/BY4 (Table 2.1). The analysis reached full discriminatory capacity (100%) with three morphological characters: SxN, SExH, and SW. Results suggested that SW and SxN are the most contributing variables to differentiate among the five groups (provinces) (Table 2.7). The coefficients of the Fisher linear discriminant function for each locality are shown in Table 2.8. The plot using the first 2 unstandardized canonical discriminant function coefficients, showed the intraspecific variation of the individuals and delimitations of the groups (Figure 2.5). Some groups

(provinces) partially overlap due to the relative high intraspecific variability, while other localities, such as AtlS/BY10 and PacS/BY8, were quite clearly defined. The classification, evaluated by using the Jack-knife validation for 5 categories among the dependent variable (provinces) showed that more than 78.7 % of the cases were correctly classified. For PacN/BY12 the model classified correctly 77.8 % of the cases, PacS/BY8 100 %, AtlN/BY4 60 %, AtlS/BY10 62.5 %, and for Med/BY4 90 % (Table 2.9).

Table 2.7. Matrix structure of CDA using morphological characters and samples classified by locality. Combined intra-group correlations between discriminating variables and canonical discriminant functions typified. Variables ordered by size of correlation with function.* Largest absolute correlation between each variable and any discriminant function. (b) This variable is not used in the analysis.

	Function		
	1	2	3
SW	,935*	,142	,325
LCD4[b]	,879*	,169	,223
SD5T[b]	,688*	-,082	,261
GCD3[b]	,658*	,209	-,123
LCD2[b]	,617*	,164	-,029
ST[b]	,585*	,061	,260
SExV[b]	,582*	,316	,527
CT[b]	,529*	,033	,249
GCD1[b]	,517*	,107	-,162
LCD3[b]	,505*	,233	,152
GCD4[b]	,499*	,257	,246
GFD[b]	,423*	,363	,090
LCD1[b]	,403*	,140	-,053
GCD2[b]	,358*	,326	-,016
α[b]	,264*	,010	-,089
L[b]	,146*	,128	,100
SxN	,515	,769*	-,378
H[b]	,168	,216*	,078
SExH	,291	,504	,813*
LFD[b]	,064	,100	,158*

Table 2.8. Coefficients of classification of the Fisher's linear discriminant functions for each locality.

	Area/ Province				
	PacN/BY12	PacS/BY8	AtlN/BY4	AtlS/BY10	Med/BY4
SxN	,268	,218	,188	,151	,167
SExH	4,076	-2,062	2,340	1,766	1,097
SW	,013	2,506	-,217	1,228	,440
(Constant)	-24,096	-42,420	-10,379	-20,282	-11,585

Table 2.9. Result of classification for each locality. Correctly classified 80.9% of original grouped cases, and 78.7% of the grouped cases validated by cross-validation. (b) Cross-validation applies only to cases of analysis, and each case is classified by the functions derived.

Code Area/Province			Predicted ownership group					Total
			PacN/BY12	PacS/BY8	AtlN/BY4	AtlS/BY10	Med/BY4	
Original	Inventory	PacN/BY12	8	0	1	0	0	9
		PacS/BY8	0	10	0	0	0	10
		AtlN/BY4	0	0	6	0	4	10
		AtlS/BY10	0	1	0	5	2	8
		Med/BY4	0	0	1	0	9	10
	%	PacN/BY12	88,9	0,0	11,1	0,0	0,0	100,0
		PacS/BY8	0,0	100,0	0,0	0,0	0,0	100,0
		AtlN/BY4	0,0	0,0	60,0	0,0	40,0	100,0
		AtlS/BY10	0,0	12,5	0,0	62,5	25,0	100,0
		Med/BY4	0,0	0,0	10,0	0,0	90,0	100,0
Crossed validation[b]	Inventory	PacN/BY12	7	0	1	1	0	9
		PacS/BY8	0	10	0	0	0	10
		AtlN/BY4	0	0	6	0	4	10
		AtlS/BY10	0	1	0	5	2	8
		Med/BY4	0	0	1	0	9	10
	%	PacN/BY12	77,8	0,0	11,1	11,1	0,0	100,0
		PacS/BY8	0,0	100,0	0,0	0,0	0,0	100,0
		AtlN/BY4	0,0	0,0	60,0	0,0	40,0	100,0
		AtlS/BY10	0,0	12,5	0,0	62,5	25,0	100,0
		Med/BY4	0,0	0,0	10,0	0,0	90,0	100,0

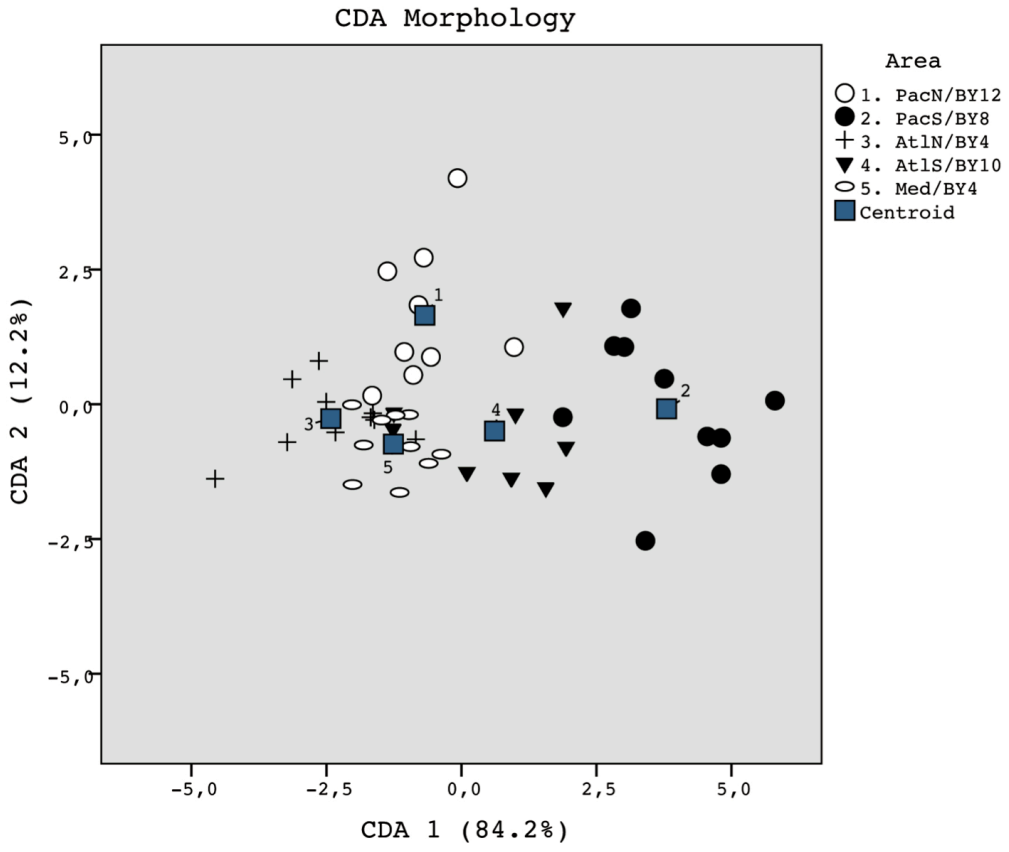


Figure 2.5. Plot of the first two functions from discriminant analyses of the partial morphological data (10 individuals per sampling site), classified by locality.

In order to investigate if morphological characters show a more general and clear biogeographical pattern, numbers of samples and marine provinces were incremented for the analysis. A total of 174 specimens of *D. dianthus* beloging to 13 provinces were used to perform the CDA (Table 2.1). No differences in the skeletal morphology of *D. dianthus* were found for the 13 provinces (Figure 2.6 and Tables 2.10, 2.11 and 2.12). Similar results were obtained for the same dataset sorted by marine areas and depth zones classification (Figure 2.7 and Tables 2.13, 2.14 and 2.15). By contrast, in size classes classification, corals were clustered as five separate groups and GD4, α, and SxN were the most contributing variables to mentioned difference. The same clear clustering was not found when samples were classified by marine provinces within each class (Figure 2.8 and Tables 2.13, 2.14 and 2.15). Pearson's correlation coefficients among L, GCD, S, and CT characters were close to zero, showing a no linear relationship (Figure 2.9).

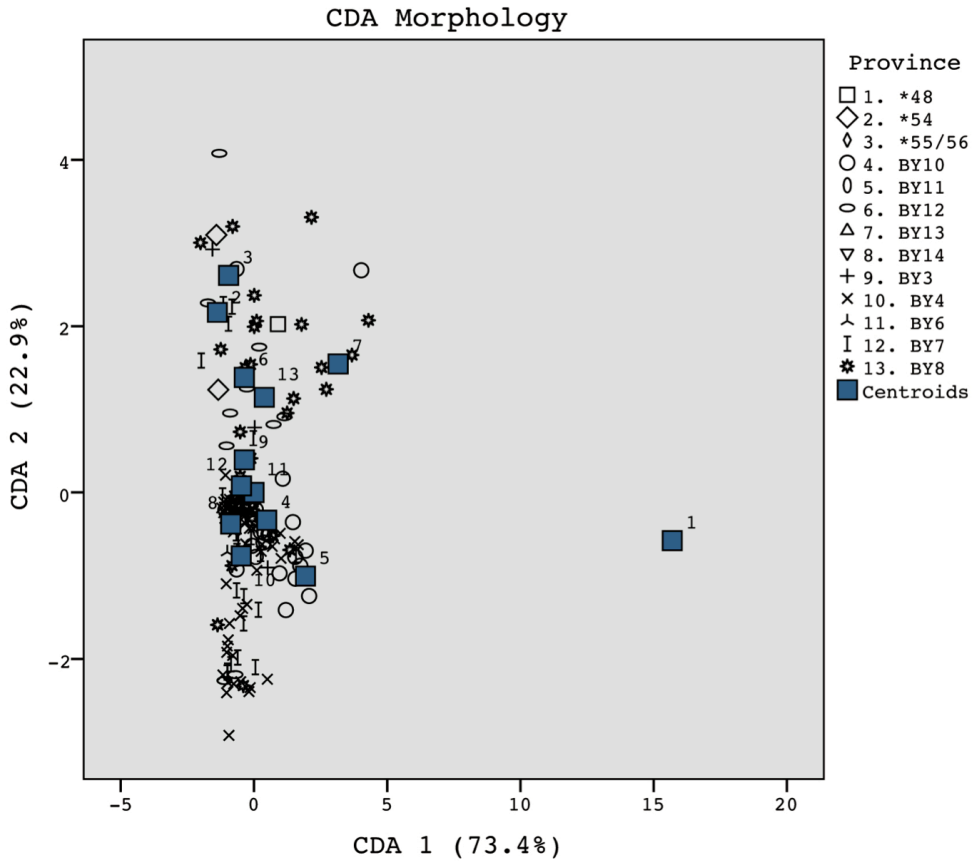


Figure 2.6. Plot of the first two functions from discriminant analyses of the all morphological data, classified by marine province. *shallow water province.

Table 2.10. Matrix structure of CDA using morphological characters and samples classified by marine province. Combined intra-group correlations between discriminating variables and canonical discriminant functions typified. Variables ordered by size of correlation with function.* Largest absolute correlation between each variable and any discriminant function. (b) This variable is not used in the analysis.

	Function		
	1	2	3
L	,788*	,308	,533
SxN	,172	,963*	,208
GCD4	,074	,450	,890*
LCD4[b]	,019	,424	,877*
SW[b]	-,046	-,020	,625*
GFD[b]	-,059	,107	,622*
SExV[b]	-,042	-,003	,562*
CT[b]	-,083	,055	,543*
LCD1[b]	-,025	,088	,535*
GCD2[b]	,050	,049	,497*
LCD2[b]	-,027	,013	,486*
LCD3[b]	-,028	,009	,465*
SD5T[b]	,036	,004	,460*
GCD3[b]	-,089	,058	,455*
SExH[b]	-,011	,071	,411*
GCD1[b]	-,166	,077	,389*
ST[b]	-,068	,029	,364*
H[b]	,164	-,001	,353*
α[b]	-,319	,227	,329*
LFD[b]	,046	-,024	,100*

Table 2.11. Coefficients of classification of the Fisher's linear discriminant functions for each marine province. *shallow water province.

	Province											
	*48	*54	*55/56	BY10	BY11	BY12	BY13	BY14	BY3	BY4	BY6	BY8
L	1,108	-,090	-,066	,068	,176	-,011	,227	-,024	,001	,008	-,002	,043
GCD4	-1,062	-,123	-,088	-,097	-,092	-,132	-,346	-,063	-,107	-,098	-,092	-,119
SxN	,172	,344	,347	,185	,125	,291	,296	,198	,237	,183	,220	,262
(Constant)	-156,038	-25,761	-29,126	-11,211	-16,313	-19,553	-32,084	-12,808	-14,892	-7,816	-12,024	-18,399

Table 2.12. Result of classification for each marine province. Correctly classified 50.6% of original grouped cases, and 40.6% of the grouped cases validated by cross-validation. (b) Cross-validation applies only to cases of analysis, and each case is classified by the functions derived. *shallow water province.

			Predicted ownership group												
Code Province			*48	*54	*55/56	BY10	BY11	BY12	BY13	BY14	BY3	BY4	BY7	BY8	Total
Original	Inventory	*48	1	0	0	0	0	0	0	0	0	0	0	1	2
		*54	0	1	0	0	0	0	0	0	0	0	1	0	2
		*55/56	0	0	0	0	0	1	0	0	0	0	0	0	1
		BY10	0	0	0	16	0	0	1	0	0	12	0	1	30
		BY11	0	0	0	1	0	0	0	0	0	0	0	0	1
		BY12	0	1	0	0	0	1	0	0	0	4	1	4	11
		BY13	0	0	0	0	0	0	1	0	0	0	0	0	1
		BY14	0	0	0	0	0	0	0	0	0	1	0	0	1
		BY3	0	0	0	0	0	1	0	0	0	3	0	1	5
		BY4	0	0	0	6	0	0	0	0	0	56	0	0	62
		BY7	0	0	0	3	0	3	0	0	0	21	1	1	29
		BY8	0	0	1	3	0	1	3	0	0	9	0	11	28
	%	*48	50,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	50,0	100,0
		*54	0,0	50,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	50,0	0,0	100,0
		*55/56	0,0	0,0	0,0	0,0	0,0	100,0	0,0	0,0	0,0	0,0	0,0	0,0	100,0
		BY10	0,0	0,0	0,0	53,3	0,0	0,0	3,3	0,0	0,0	40,0	0,0	3,3	100,0
		BY11	0,0	0,0	0,0	100,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	100,0
		BY12	0,0	9,1	0,0	0,0	0,0	9,1	0,0	0,0	0,0	36,4	9,1	36,4	100,0
		BY13	0,0	0,0	0,0	0,0	0,0	0,0	100,0	0,0	0,0	0,0	0,0	0,0	100,0
		BY14	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	100,0	0,0	0,0	100,0
		BY3	0,0	0,0	0,0	0,0	0,0	20,0	0,0	0,0	0,0	60,0	0,0	20,0	100,0
		BY4	0,0	0,0	0,0	9,7	0,0	0,0	0,0	0,0	0,0	90,3	0,0	0,0	100,0
		BY7	0,0	0,0	0,0	10,3	0,0	10,3	0,0	0,0	0,0	72,4	3,4	3,4	100,0
		BY8	0,0	0,0	3,6	10,7	0,0	3,6	10,7	0,0	0,0	32,1	0,0	39,3	100,0
Crossed validation[b]	Inventory	*48	0	0	0	0	0	0	1	0	0	0	0	1	2
		*54	0	0	0	0	0	1	0	0	0	0	1	0	2
		*55/56	0	0	0	0	0	1	0	0	0	0	0	0	1
		BY10	0	0	0	16	0	0	1	0	0	12	0	1	30
		BY11	0	0	0	1	0	0	0	0	0	0	0	0	1
		BY12	0	1	0	0	0	1	0	0	0	4	1	4	11
		BY13	0	0	0	0	0	0	0	0	0	0	0	1	1
		BY14	0	0	0	0	0	0	0	0	0	1	0	0	1
		BY3	0	0	0	0	0	1	0	0	0	3	0	1	5
		BY4	0	0	0	6	0	0	0	0	0	56	0	0	62
		BY7	0	1	0	3	0	2	0	0	0	21	0	2	29
		BY8	0	0	1	5	0	3	3	0	0	9	0	7	28
	%	*48	0,0	0,0	0,0	0,0	0,0	0,0	50,0	0,0	0,0	0,0	0,0	50,0	100,0
		*54	0,0	0,0	0,0	0,0	0,0	50,0	0,0	0,0	0,0	0,0	50,0	0,0	100,0
		*55/56	0,0	0,0	0,0	0,0	0,0	100,0	0,0	0,0	0,0	0,0	0,0	0,0	100,0
		BY10	0,0	0,0	0,0	53,3	0,0	0,0	3,3	0,0	0,0	40,0	0,0	3,3	100,0
		BY11	0,0	0,0	0,0	100,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	100,0
		BY12	0,0	9,1	0,0	0,0	0,0	9,1	0,0	0,0	0,0	36,4	9,1	36,4	100,0
		BY13	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	100,0	100,0
		BY14	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	100,0	0,0	0,0	100,0
		BY3	0,0	0,0	0,0	0,0	0,0	20,0	0,0	0,0	0,0	60,0	0,0	20,0	100,0
		BY4	0,0	0,0	0,0	9,7	0,0	0,0	0,0	0,0	0,0	90,3	0,0	0,0	100,0
		BY7	0,0	3,4	0,0	10,3	0,0	6,9	0,0	0,0	0,0	72,4	0,0	6,9	100,0
		BY8	0,0	0,0	3,6	17,9	0,0	10,7	10,7	0,0	0,0	32,1	0,0	25,0	100,0

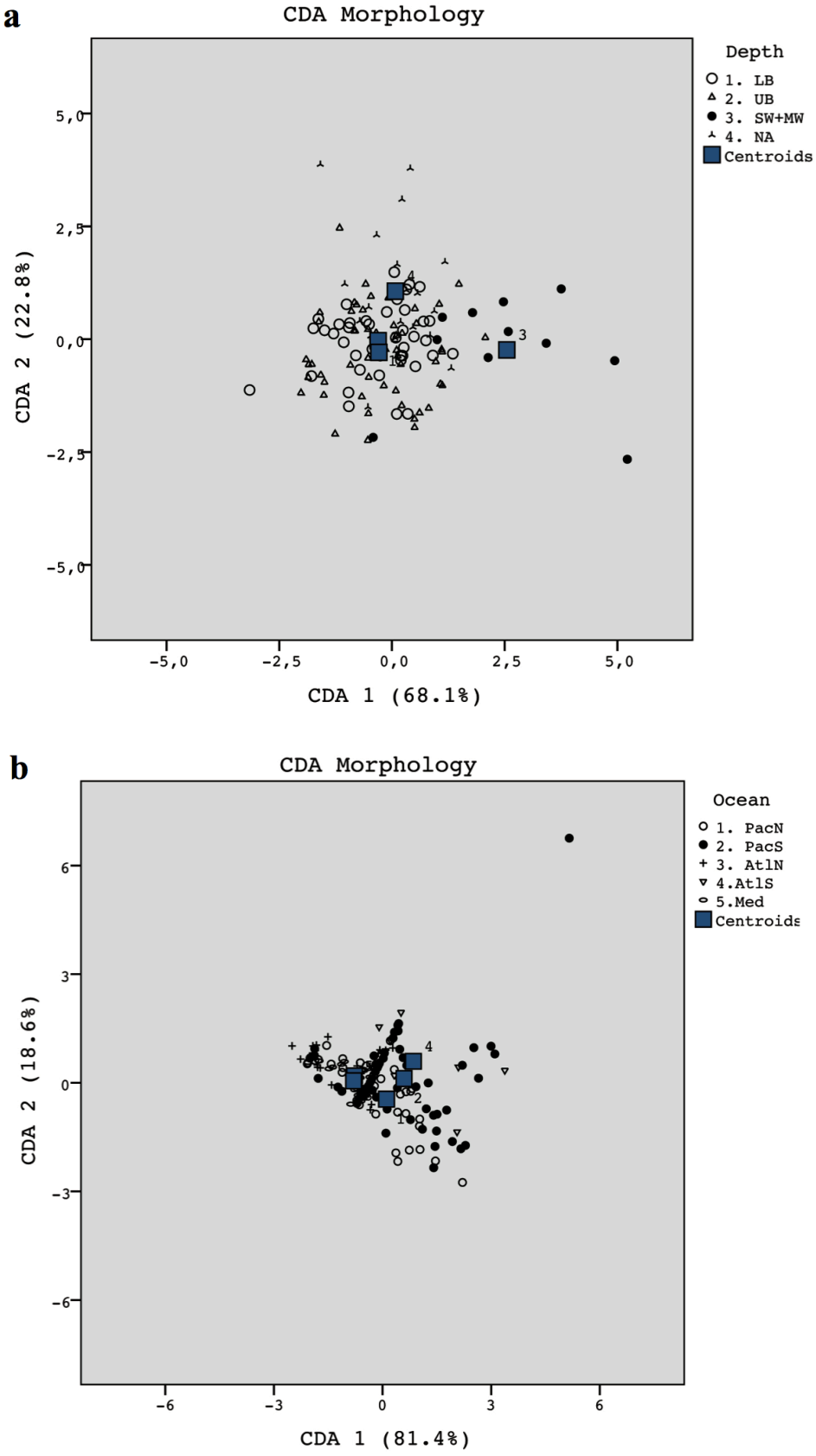


Figure 2.7. Plots of the first two functions from discriminant analyses of the all morphological data, classified by depth (a) and marine regions (b).

Table 2.13. Matrix structure of CDA using morphological characters and samples classified by size class. Combined intra-group correlations between discriminating variables and canonical discriminant functions typified. Variables ordered by size of correlation with function.* Largest absolute correlation between each variable and any discriminant function. (b) This variable is not used in the analysis.

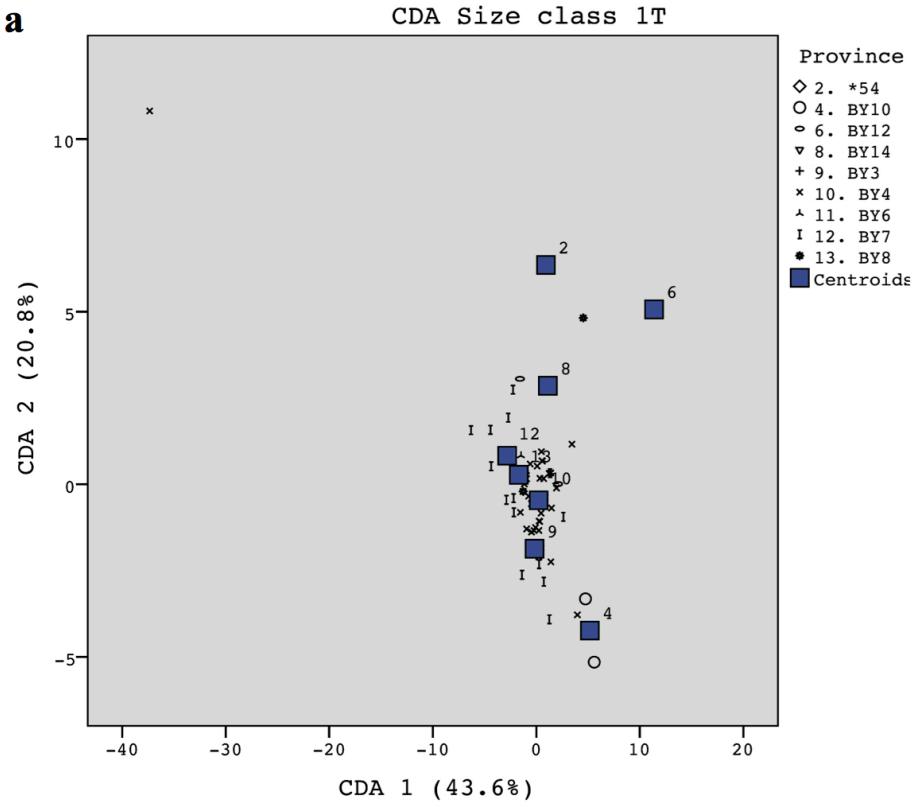
	Function		
	1	2	3
GCD4	,804*	,420	,421
LCD4[b]	,791*	,401	,368
CT[b]	,111	,482*	,339
SW[b]	,261	,444*	,297
GFD[b]	,323	,423*	,252
SExV[b]	,220	,403*	,291
SD5T[b]	,094	,369*	,176
LCD3[b]	,176	,303*	,237
ST[b]	,080	,292*	,196
GCD2[b]	,232	,290*	,174
α	-,045	,320	,946*
SxN	,604	-,484	,633*
GCD1[b]	,124	,192	,451*
LCD1[b]	,272	,287	,396*
SExH[b]	,206	,204	,307*
GCD3[b]	,178	,246	,279*
H[b]	,276	,142	-,278*
LCD2[b]	,173	,269	,276*
LFD[b]	-,005	,023	-,148*

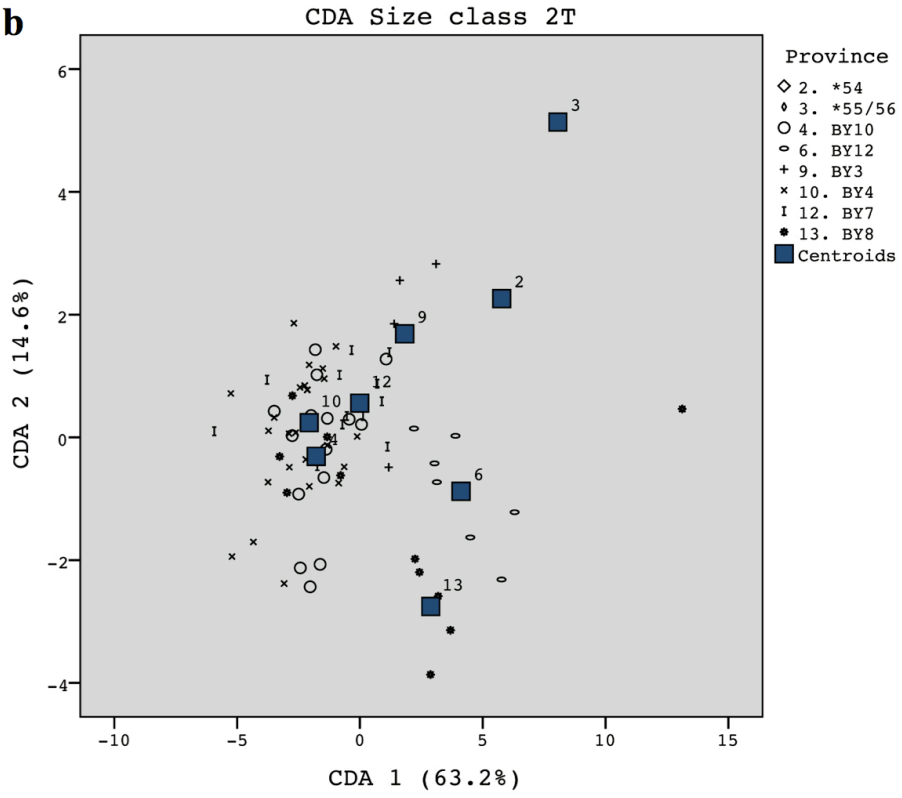
Table 2.14. Coefficients of classification of the Fisher's linear discriminant functions for each size class.

	Code size class				
	1T	2T	3T	4T	>5T
GCD4	-,098	,003	,170	,162	-,056
α	,073	,035	-,001	-,071	-,077
SxN	,134	,155	,140	,237	,276
(Constant)	-7,844	-9,957	-14,067	-25,048	-23,964

Table 2.15. Result of classification for each size class. Correctly classified 68.4% of original grouped cases, and 66.1 % of the grouped cases validated by cross-validation. (b) Cross-validation applies only to cases of analysis, and each case is classified by the functions derived.

Code Size Class			Predicted ownership group					Total
			1T	2T	3T	4T	>5T	
Original	Inventory	1T	50	15	0	0	0	65
		2T	15	52	6	1	0	74
		3T	0	13	9	2	0	24
		4T	0	2	0	5	0	7
		>5T	0	0	0	0	1	1
	%	1T	76,9	23,1	0,0	0,0	0,0	100,0
		2T	20,3	70,3	8,1	1,4	0,0	100,0
		3T	0,0	54,2	37,5	8,3	0,0	100,0
		4T	0,0	28,6	0,0	71,4	0,0	100,0
		>5T	0,0	0,0	0,0	0,0	100,0	100,0
Crossed validation[b]	Inventory	1T	49	16	0	0	0	65
		2T	14	51	6	2	1	74
		3T	1	12	9	2	0	24
		4T	0	2	1	4	0	7
		>5T	0	1	0	0	0	1
	%	1T	75,4	24,6	0,0	0,0	0,0	100,0
		2T	18,9	68,9	8,1	2,7	1,4	100,0
		3T	4,2	50,0	37,5	8,3	0,0	100,0
		4T	0,0	28,6	14,3	57,1	0,0	100,0
		>5T	0,0	100,0	0,0	0,0	0,0	100,0


a
CDA Size class 1T
Province
2. *54
4. BY10
6. BY12
8. BY14
9. BY3
10. BY4
11. BY6
12. BY7
13. BY8
Centroids
CDA 2 (20.8%)
CDA 1 (43.6%)
10
5
0
-5
-40
-30
-20
-10
0
10
20


b
CDA Size class 2T
Province
2. *54
3. *55/56
4. BY10
6. BY12
9. BY3
10. BY4
12. BY7
13. BY8
Centroids
CDA 2 (14.6%)
CDA 1 (63.2%)
6
4
2
0
-2
-4
-10
-5
0
5
10
15

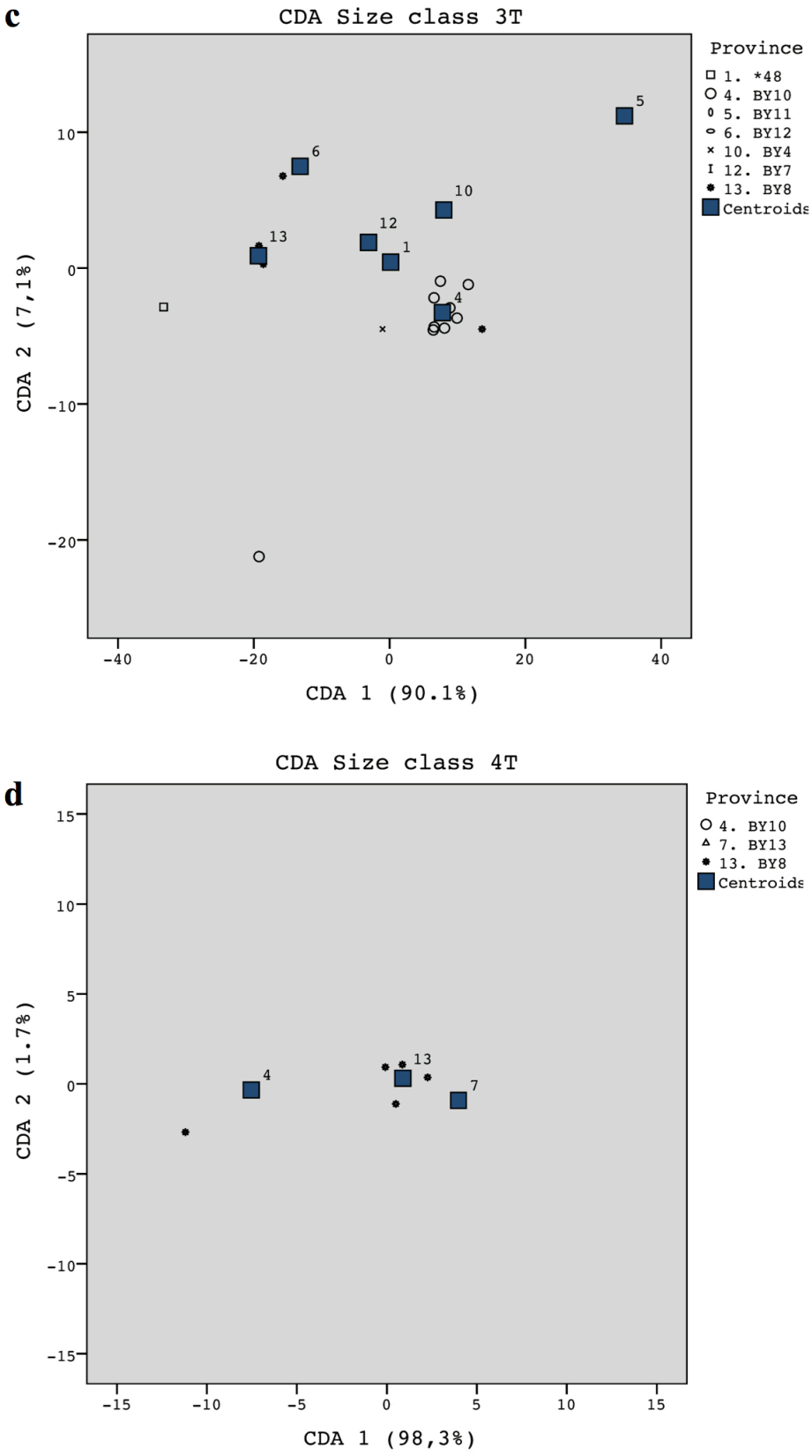


Figure 2.8. Plots of the first two functions from discriminant analyses of the entire morphological data, classified by size class: 1st tridecade (a), 2nd tridecade (b), 3rd tridecade (c) and 4th tridecade (d). *shallow water province.

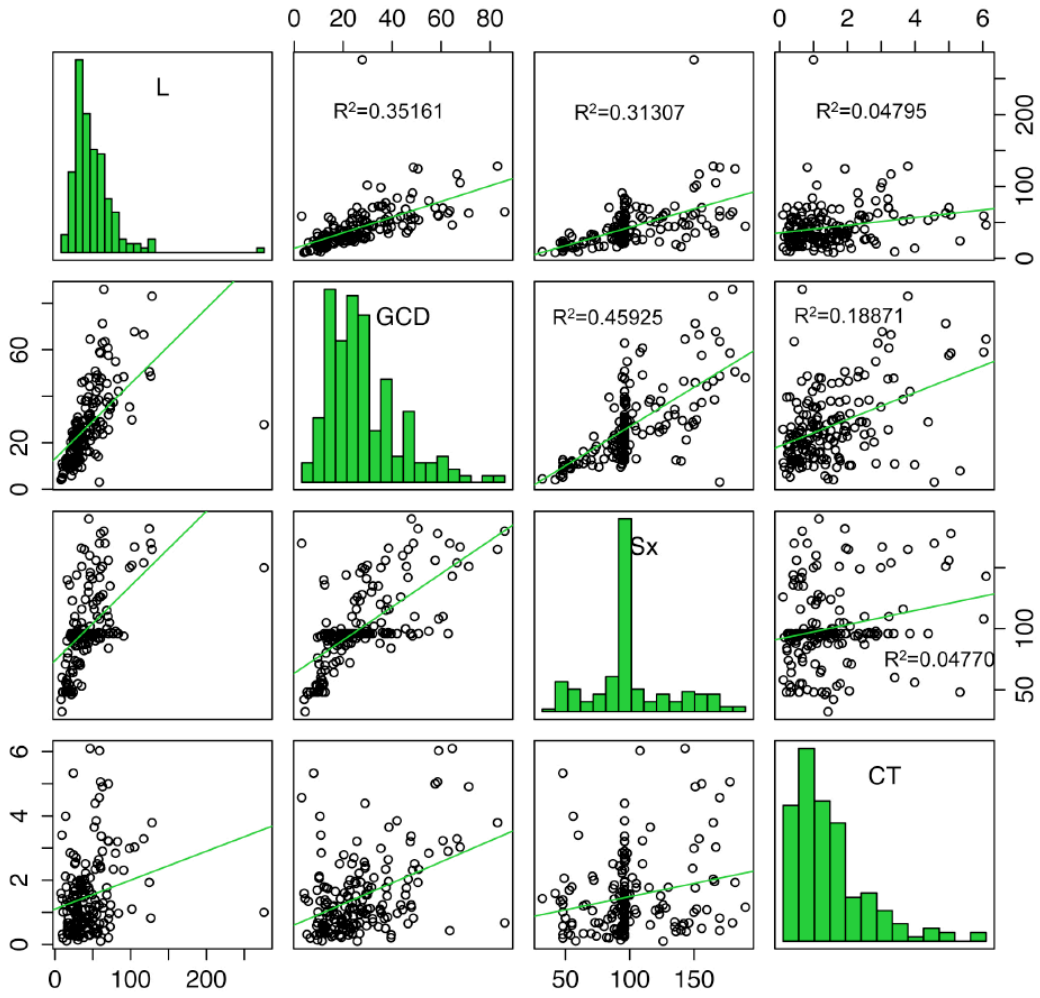


Figure 2.9. Scatterplots and Pearson's correlation coefficients of following morphometric parameters: L, GCD, Sx and CT.

Skeletal analysis: 3D coordinates of landmarks

For each corallum, sets of landmarks along corallum wall could not be possible measured adequately, due to the impracticability in defining reliably homologous structures. Instead, 19 landmarks along costosepta were successfully found (Figure 2.3) and a total of 44 pairs of landmarks endpoints (22 per side) (Table 2.16) were used to perform the corresponding analysis. Although a slight structure throughout the oceans is visible in the graphs (Figures 2.10 and 2.11), PCA and CVA analyses of data set using individuals and provinces as *a priori* groups found no significant differences in landmarks endpoints between individuals and provinces, respectively, as well as not associated axes positively correlated with specific pair endpoints (Tables 2.17, 2.18 and 2.19).

Table 2.16. 3D Landmarks on corallites of *D. dianthus*; endpoint landmarks correspond to Fig 19. A total of 22 pairs of endpoints are referred to where distances were measured in 3 dimensional spaces.

No	Pair of endpoints	Description	Detailed description
1	1-3	(septa height)	The intercept point between septa and wall on left side of 4th septa and wall to the top outermost point
2	2-5	(distance between septa)	The intercept point between septa and wall on left side of 4th septa to the intercept point between septa and wall on right side of 5th septa (distance between 4th septa and 5th septa)
3	3-4	(septa length)	On 4th septa: length from the endpoint of 4th septa to top outermost point (length of 4th septa)
4	3-2	(septa height)	The top outermost point to the intercept point between septa and wall on rigth side of 4th septa and wall
5	5-6	(septa height)	The intercept point between septa and wall on left side of 5th septa and wall to the top outermost point
6	6-7	(septa length)	On 5th septa: length from the endpoint of 5th septa to top outermost point (length of 5th septa)
7	6-8	(septa height)	The top outermost point to the intercept point between 5th septa and wall on left side of 2nd septa and wall
8	8-11	(septa height)	The intercept point between 2nd septa and 5th septa on right side of 2nd septa and wall to the top outermost point
9	9-12	(septa length)	On 2nd septa: length from the top middle point of intercept line between septa and wall on left side of 5th septa and septa and wall on right side of 5th septa to the endpoint of 2nd septa (partial length of 2nd septa)
10	10-13	(septa height)	The top outermost point to the intercept point between 5th septa and wall on rigth side of 2nd septa and wall
11	11-9	(septa length)	On 2nd septa: length from the outermost point of 2nd septa to top middle point of intercept line between septa and wall on left side of 5th septa and septa and wall on right side of 5th septa (partial length of 2nd septa)
12	11-10	(septa height)	The top outermost point to the intercept point between 2nd septa and wall on left side of 5th septa and wall
13	13-14	(septa length)	On 5th septa: length from the endpoint of 5th septa to top outermost point (length of 5th septa)
14	13-15	(septa height)	The top outermost point to the intercept point between septa and wall on rigth side of 5th septa and wall
15	15-16	(distance between septa)	The intercept point between septa and wall on left side of 5th septa to the intercept point between septa and wall on right side of 4th septa (distance between 5th septa and 4th septa)
16	16-18	(septa height)	The intercept point between septa and wall on left side of 4th septa and wall to the top outermost point
17	18-17	(septa height)	The top outermost point to the intercept point between septa and wall on rigth side of 4th septa and wall
18	18-19	(septa length)	On 4th septa: length from the endpoint of 4th septa to top outermost point (length of 4th septa)
19	16-17	(septa width)	The intercept point between septa and wall on left side of 4th septa to the intercept point between septa and wall on right side of 4th septa (4th septa thickness)
20	8-10	(septa width)	On 2nd septa: the intercept point between septa and 5th septa on right side to intercept point between septa and 5th septa on left side (2nd septa thickness)
21	11-12	(septa length)	On 2nd septa: length from the endpoint of 2nd septa to top outermost point (length of 2nd septa)
22	1-2	(septa width)	On 4th septa: the intercept point between septa and wall on left side to intercept point between septa and wall on right side (4th septa thickness)

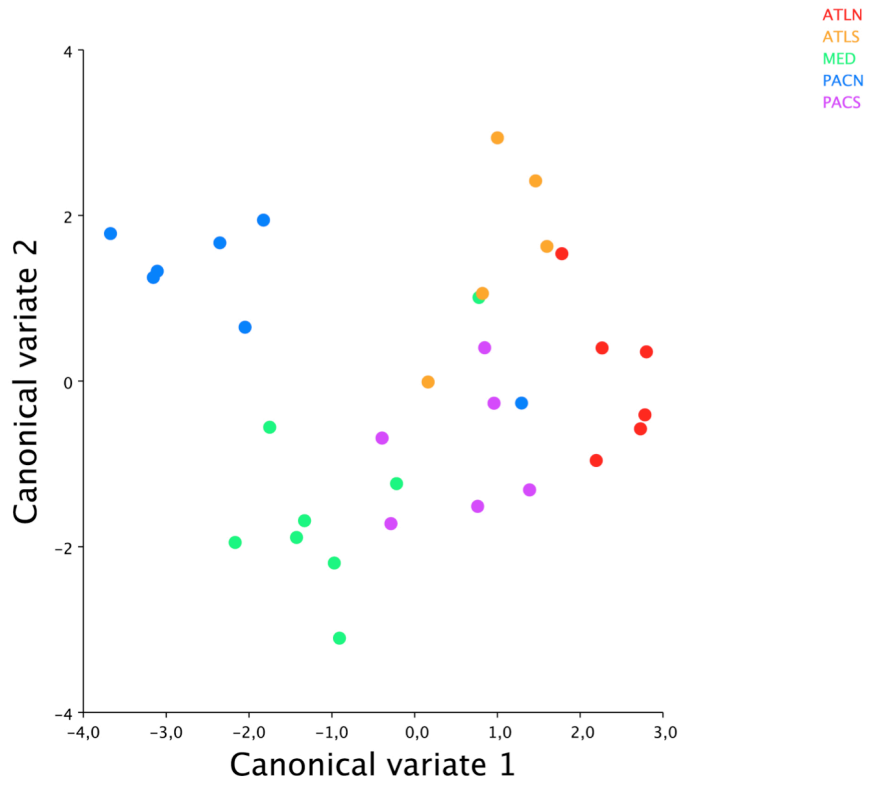


Figure 2.10. Plots of the first two functions from discriminant analyses of 3D landmarks data in *Desmophyllum dianthus* individuals, classified by locality.

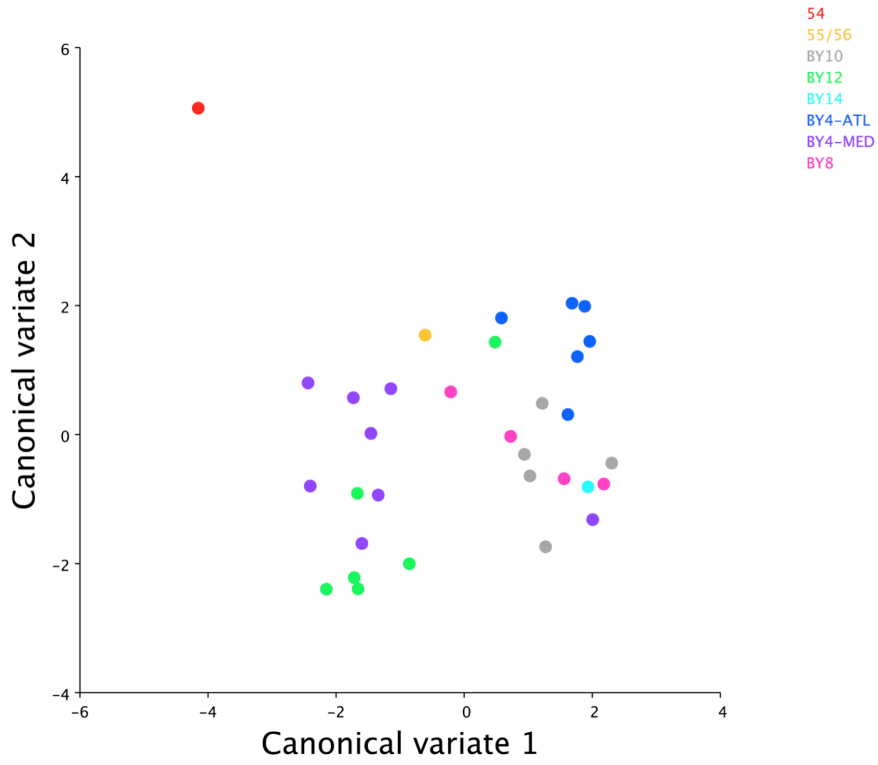


Figure 2.11. Plots of the first two functions from discriminant analyses of 3D landmarks data in *Desmophyllum dianthus* individuals, classified by marine province.

Table 2.17. Summary of statistics of PCA. The dataset contains 32 observations, of which 32 are included for analyses, and 38 landmarks in 3 dimensions.

Principal Component Analysis (PCA)

	Eigenvalues	% Variance	Cumulative %
1.	0,00492701	20,508	20,508
2.	0,00321907	13,399	33,907

Total variance: 0,02402494

Table 2.18. Summary of statistics of CVA with samples classified by locality. The dataset contains 32 observations, of which 32 are included for analyses, and 38 landmarks in 3 dimensions.

Canonical Variate Analysis (CVA)

Classification criterion: Locality

Groups	Locality	Observations
1.	AtlN	6
2.	AtlS	5
3.	Med	8
4.	PacN	7
5.	PacS	6

Variation among groups, scaled by the inverse of the within-group variation

	Eigenvalues	% Variance	Cumulative %
1.	3,02708236	42,903	42,903
2.	1,63206687	23,132	66,035

Mahalanobis distances among groups

	AtlN	AtlS	Med	PacN
AtlN	3,7381			
AtlS	4,1672	3,9175		
Med	4,7718	4,1992	3,4043	
PacN	3,4954	3,7553	3,2724	3,873

Procrustes distances among groups

	AtlN	AtlS	Med	PacN
AtlN	0,1215			
AtlS	0,1106	0,0803		
Med	0,1027	0,0793	0,0545	
PacN	0,0973	0,0935	0,0777	0,0808

Table 2.19. Summary of statistics of CVA with samples classified by marine province. The dataset contains 32 observations, of which 32 are included for analyses, and 38 landmarks in 3 dimensions.

Canonical Variate Analysis (CVA)

Classification criterion: Province

Groups	Province	Observations
1.	*54	1
2.	*55/56	1
3.	BY10	5
4.	BY12	6
5.	BY14	1
6.	BY4-ATL	6
7.	BY4-MED	8
8.	BY8	4

Variation among groups, scaled by the inverse of the within-group variation

	Eigenvalues	% Variance	Cumulative %
1.	3,01444651	30,707	30,707
2.	2,33320581	23,767	54,474

Mahalanobis distances among groups

	*54	*55/56	BY10	BY12	BY14	BY4-ATL	BY4-MED
55/56	6,4893						
BY10	8,0751	4,891					
BY12	7,5128	4,6469	3,8829				
BY14	9,6594	5,6578	5,5186	5,7333			
BY4-ATL	7,4597	4,2884	3,5043	4,2093	5,4404		
BY4-MED	6,8049	3,8902	3,5348	2,6029	5,7703	3,7854	
BY8	8,0058	4,3852	3,5956	3,9141	5,4452	3,5079	3,4642

Procrustes distances among groups

	*54	*55/56	BY10	BY12	BY14	BY4-ATL	BY4-MED
55/56	0,1936						
BY10	0,1945	0,1226					
BY12	0,1798	0,1326	0,0824				
BY14	0,2468	0,1789	0,1573	0,1611			
BY4-ATL	0,1938	0,123	0,1043	0,1094	0,156		
BY4-MED	0,1871	0,1202	0,075	0,054	0,1598	0,1106	
BY8	0,2047	0,1501	0,0914	0,0909	0,1703	0,1283	0,0909

Tissue analysis: characterization of the cnidom

The following results are based on the study of over 24.000 undischarged capsules. The obtained diversity of cnidae in *D. dianthus* included 12 categories: SP, two H, three B, and five MpM. The diversity and distribution of cnidae in the five stations studied were compared (Table 2.20). Their morphology (types and categories) was also represented (Figures 2.12, 2.13, 2.14 and 2.16). The cnidome composition and size of cnidae present in differents tissue were found to be quite constant throughout the sampling areas. Eleven categories were common in the five stations studied here: Bs1 and MpM (scapus); Sp, H1, and MpM3 (tentacles); Bs2, Bs3, and MpM1 (pharynx); Bs2, MpM2, and MpM4 (mesenterial filaments). In addition, every locality also showed lack of cnidae catergories per tissue: AtlN/BY4 (Bs3T and MpM1T); AtlS/BY10 (MpM2S, Bs1T, MpM1T, Bs1P, and MpM3P); PacN/BY12 (Bs3T and MpM1T); PacS/48 (Bs1T and Bs2T); and Med/BY4 (Bs1T, MpM1T, Bs1P, MpM3P, H2MF, MpM1MF, and MpM5MF) (Table 2.20).

Table 2.20. Cnydocysts of *Desmophyllum dianthus* from the five localities. Mean (M), standard deviation (SD) and range. For abbreviations of cnidae see the main text.

		*D. dianthus*_AtlS/BY10	D. dianthus_AtlN/BY4	*D. dianthus*_PacN/BY12	*D. dianthus*_PacS/48	*D. dianthus*_ Med/BY4
Tissue	**Cnidae**	**M ± SD (Range)**	**M ± SD (Range)**	**M ± SD (Range)**	**M ± SD (Range)**	**M ± SD (Range)**
SCAPUS	Bs1	9,8 ± 1,1 x 3,0 ± 0,0 (8,0 -12,0) x (3)	14,9 ± 3,1 x 5,6 ± 0,5 (7,2 - 23,8 x 2,1 - 4,8)	13,4 ± 4,7 x 3,1 ± 0,4 (6,3 - 21,9) x (2,2 - 4,3)	11,5 ± 3,5 x 2,8 ± 0,3 (7,8 - 19) x (2,2 - 3,5)	16,9 ± 2,6 x 3,0 ± 0,1 (10,0 - 20,0) x (2,5 - 3,0)
	Mp-M1	18,3 ± 2,7 x 6,5 ± 0,9 (15,0 - 26,0) x (5,0 - 9,0)	18 ± 2,5 x 7,3 ± 1,7 (13,3-24,1) x (4,7-10)	16,7 ± 1,7 x 6,9 ± 0,6 (13,3 - 20) x (5,7 - 8)	16,4 ± 1,6 x 6,3 ± 0,9 (10,7-19,2) x (2,9-8,3)	13,3 ± 1,2 x 4,7 ± 0,4 (10,0 - 15,0) x (4,0 - 5,0)
	Mp-M2		32,9 ± 9,6 x 5,6 ± 0,8 (18,7-54) x (4,2-7,9)	32,2 ± 6,5 x 5,6 ± 0,9 (25,3 - 54,1) x (4,7 - 8,9)	25,0 ± 6,6 x 5,4 ± 1,1 (15,6 - 51,7) x (3,4 - 8,7)	27,8 ± 3,2 x 5,1 ± 0,3 (18,0 - 40,0) x (5,0 - 6,0)
TENTACLES	Sp	38,7 ± 7,0 x 4,5 ± 0,80 (22,0 - 55,0) x (3,0 - 6,0)	36,38 ± 7,24 x 4,18 ± 0,80 (20,5 - 48) x (2,6-6,1)	36,7 ± 7,6 x 4,9 ± 0,9 (21,4 - 48,8) x (2,7 - 6,5)	37,1 ± 8 x 4,7 ± 1,3 (22,1-56,6) x (2,4-7,3)	34,7 ± 6,2 x 4,6 ± 0,9 (22,0 - 49,0) x (3,0 - 6,0)
	Bs1		10,2 ± 1,4x 2,6 ± 0,3 (7,3-14) x (1,9-3,2)	9,6 ± 0,9 x 2,8 ± 0,4 (7,1 - 11,6) x (2 - 4,2)		
	Bs3	41,3 ± 3,1 x 4,1 ± 0,3 (33,0 - 50,0) x (4,0 - 5,0)			42,8 ± 3,6 x 5 ± 0,5 (33,5 - 55,8) x (4 - 6,4)	30,6 ± 4,1 x 4,9 ± 0,3 (20,0 - 45,0) x (3,5 - 5,0)
	H1	55,7 ± 2,7 x 19,6 ± 0,8 (48,0 - 62,0) x (16,0 - 20,0)	56,2 ± 6,2 x 18,2 ± 1,2 (45,8-69,5) x (15,4-21,1)	50,2 ± 4,2 x 18,6 ± 1,8 (41 - 56,9) x (13,8 - 22,1)	53 ± 3,19 x 17,93 ± 1,7 (47-58,5) x (14,3-22)	53,0 ± 2,1 x 20,0 ± 0,2 (50,0 - 58,0) x (19,0 - 20,0)
	Mp-M1					
	Mp-M3	45,3 ± 3,6 x 5,7 ± 0,6 (38,0 - 53,0) x (5,0 - 7,0)	46,8 ± 4,2 x 6,5 ± 0,5 (39,2-58,4) x (5,4-7,4)	49,2 ± 5,3 x 6 ± 0,5 (30,4 - 57,9) x (4,8 - 7,1)	40,7 ± 8,6 x 5,6 ± 0,7 (17,4 - 51,2) x (4,2 - 7,4)	38,32 ± 6,1 x 5,4 ± 0,6 (28,0 - 50,0) x (4,0 - 7,0)
PHARYNX	Bs1		9,1 ± 1,4 x 2,8 ± 0,3 (6,3 - 13,1) x (2,2 - 3,9)	9,3 ± 1,3 x 2,7 ± 0,4 (6,7 - 12) x (2,1 - 4,2)	8,7 ± 1,2 x 2,5 ± 0,3 (6,4 - 13,2) x (1,9 - 3,2)	
	Bs2	22,7 ± 4,3 x 3,37 ± 0,54 (15,0 -32,0) x (3,0 - 5,0)	22,2 ± 3,1 x 3,8 ± 0,4 (17,4 - 31,6) x (2,9 - 4,7)	18,5 ± 1,8 x 3,8 ± 0,6 (14,9 - 23) x (3 - 5,6)	20,4 ± 2,9 x 3,3 ± 0,4 (15,2 - 27,8) x (2,4 - 4,8)	23,0 ± 5,5 x 3,8 ± 0,4 (15,0 - 32,0) x (3,0 - 4,5)
	Bs3	45,8 ± 5,6 x 4,7 ± 0,5 (37,0 - 60,0) x (4 - 5,5)	41,3 ± 5,2 x 4,7 ± 0,6 (26,7-51,9) x (3,3-6,3)	40,8 ± 4,9 x 4,6 ± 0,5 (29,8 - 53,3) x (3,4 - 6)	38,4 ± 5,8 x 3,7 ± 0,4 (25,1 - 53,4) x (3 - 5,3)	41,3 ± 5,7 x 4,5 ± 0,5 (33,0 - 60,0) x (4,0 - 5,5)
	Mp-M1	18,0 ± 2,4 x 6,5 ± 0,6 (14,0 - 25,0) x (5,0 - 8,0)	16,4 ± 2,2 x 5,7 ± 1,4 (11,6-21,8) x (3,6-9)	17,2 ± 2,2 x 6,4 ± 1 (14,2-21,4) x (4,2-8,8)	16,2 ± 1,7 x 5,1 ± 1 (12,1 - 21,3) x (3,8 - 7,2)	17,7 ± 2,1 x 5,3 ± 0,6 (13,0 -23,0) x (4,0 - 6,5)
	Mp-M3		38 ± 7,7 x 5,9 ± 0,6 (15,2-61,6) x (3,8-7)	46,7 ± 5,9 x 5,9 ± 0,5 (32,5 - 57,8) - (5 - 7,1)	30,8 ± 8,7 x 5,6 ± 0,8 (17,7 - 51,7) x (3,4 - 7)	
MESENTERIAL FILAMENTS	Bs1	18,9 ± 2,0 x 3,5 ± 0,5 (15,0 - 22,0) x (3,0 - 4,0)	14,4 ± 3,5 x 3 ± 0,6 (6,7 - 24,4) x (2-4)	14 ± 4,9 x 3,2 ± 0,6 (6,3 - 22,8) x (2 - 4)	11,8 ± 4,7 x 2,7 ± 0,5 (6,9 - 20,5) x (1,8 - 3,6)	14,7 ± 2,2 x 3,1 ± 0,3 (12,0 -23,0) x (3,0-4,5)
	H2	105,7 ± 5,0 x 21,1 ± 2,0 (92,0 - 115,0) x (19-22)	107,7 ± 8,6 x 20,2 ± 1,5 (90,1 - 123,4) x (16,3 - 24,5)	99,1 ± 9,7 x 22,9 ± 2,5 (73,9-119,8) x (13,8-29,6)	92,1 ± 7,4 x 22,6 ± 1,9 (74 - 104,9) x (18,8 - 27,9)	
	Mp-M1	18,6 ± 2,1 x 6,6 ± 0,7 (13,0 - 23,0) x (5,0 - 8,0)	17,7 ± 2,4 x 7,4 ± 0,9 (12,4 - 23,3) x (5,1 - 9,8)	16,2 ± 1,5 x 7 ± 0,7 (13,7 - 19,3) x (5,3 - 8,2)	16,3 ± 1,6 x 6,4 ± 0,9 (12,6 - 19,1) x (4,1 - 9,7)	
	Mp-M2	30,2 ± 2,0 x 5,6 ± 0,5 (26,0 - 35,0) x (5,0 - 6,0)	28,8 ± 3,3 x 5,6 ± 0,6 (15,7 - 35,7) x (3,5 - 6,8)	28,5 ± 6 x 5,6 ± 1,2 (15,9- 48,1) x (3,2-8,4)	26,7 ± 2,9 x 5,7 ± 0,8 (22,7 - 38) x (4 - 7,6)	24,8 ± 5,3 x 5,4 ± 0,5 (15,0 - 40,0) x (5,0 - 6,0)
	Mp-M4	81,7 ± 5,5 x 10,7 ± 0,9 (65,0 - 90,0) x (10, 0-12,0)	67,3 ± 7,1 x 10,2 ± 1 (51,3-78,7) x (8,5-13,9)	63,4 ± 7,4 x 9,9 ± 1,2 (50,5-79,5) x (6,5-12,5)	55,7 ± 4,8 x 11,1 ± 1,5 (50,1 - 66,9) x (8,4 - 14,5)	73,3 ± 13,9 x 11,3 ± 1,3 (60,0 - 89,0) x (10,0 - 13,0)
	Mp-M5	129,0 ± 8,7 x 15,5 ± 3,6 (105,0 - 140,0) x (12,0 - 20,0)	117,3 ± 10 x 13, 3 ± 0,8 (83,3-138,2) x (11,5-15)	110,7 ± 16,2 x 12,7 ± 1,6 (82,4 - 142,8) x (5,5 -15,2)	104,1 ± 10,7 x 12,5 ± 1,4 (80,4 - 120,6) x (10,4 - 16,4)	

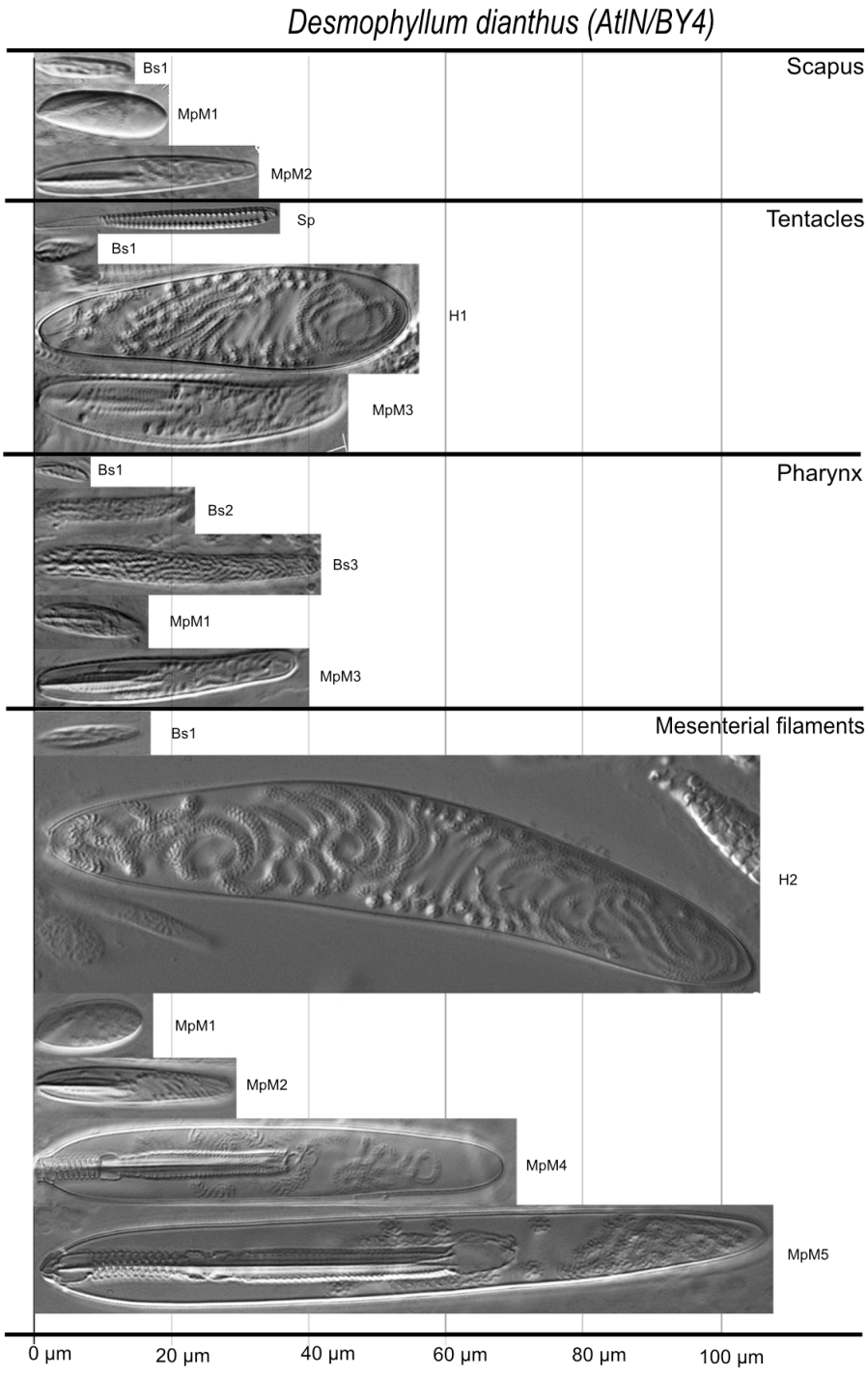


Figure 2.12. Cnidocysts of *Desmophyllum dianthus* from the North Atlantic Ocean (AtlN).

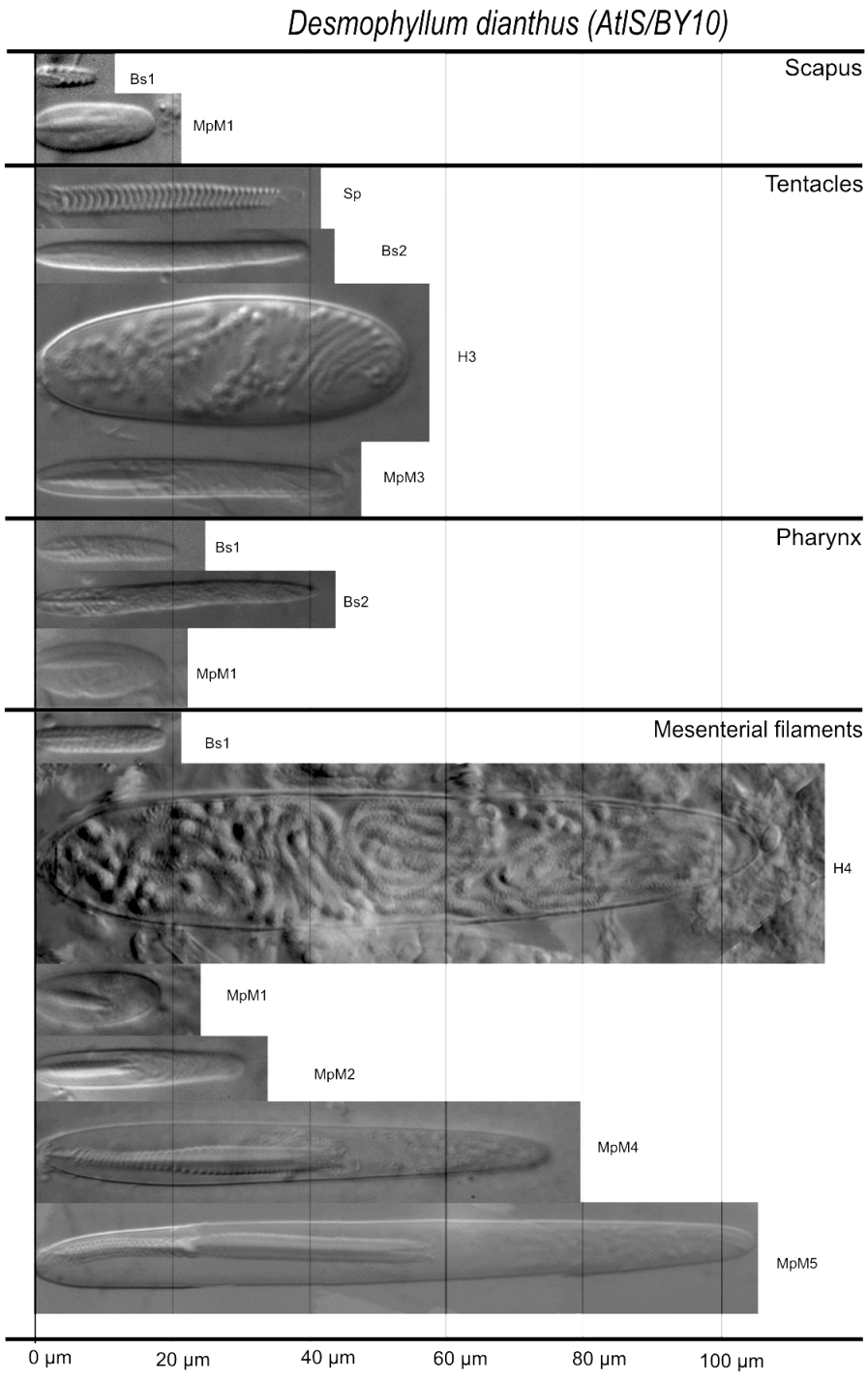


Figure 2.13. Cnidocysts of *Desmophyllum dianthus* from the South Atlantic Ocean (AtlS).

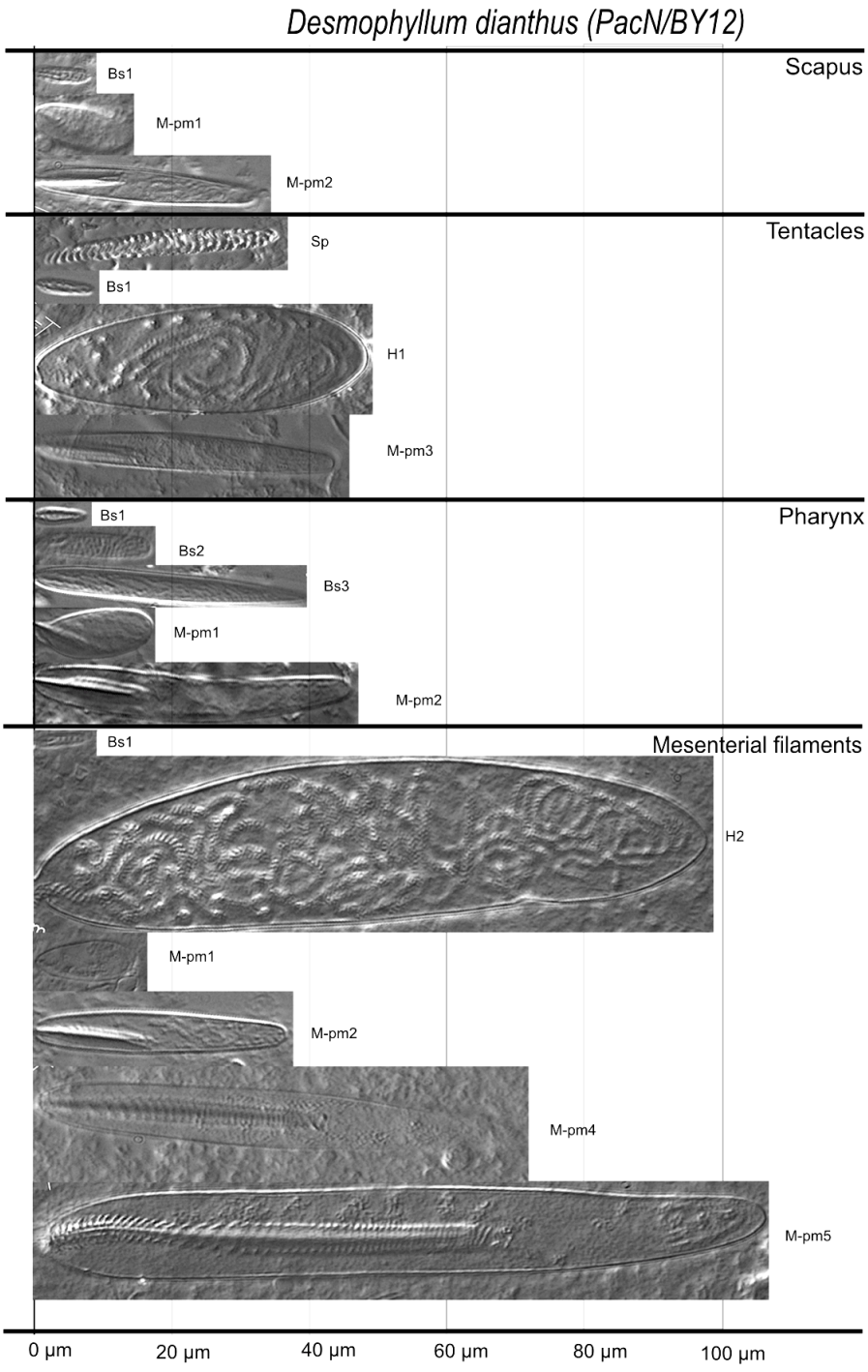


Figure 2.14. Cnidocysts of *Desmophyllum dianthus* from the North Pacific Ocean (PacS).

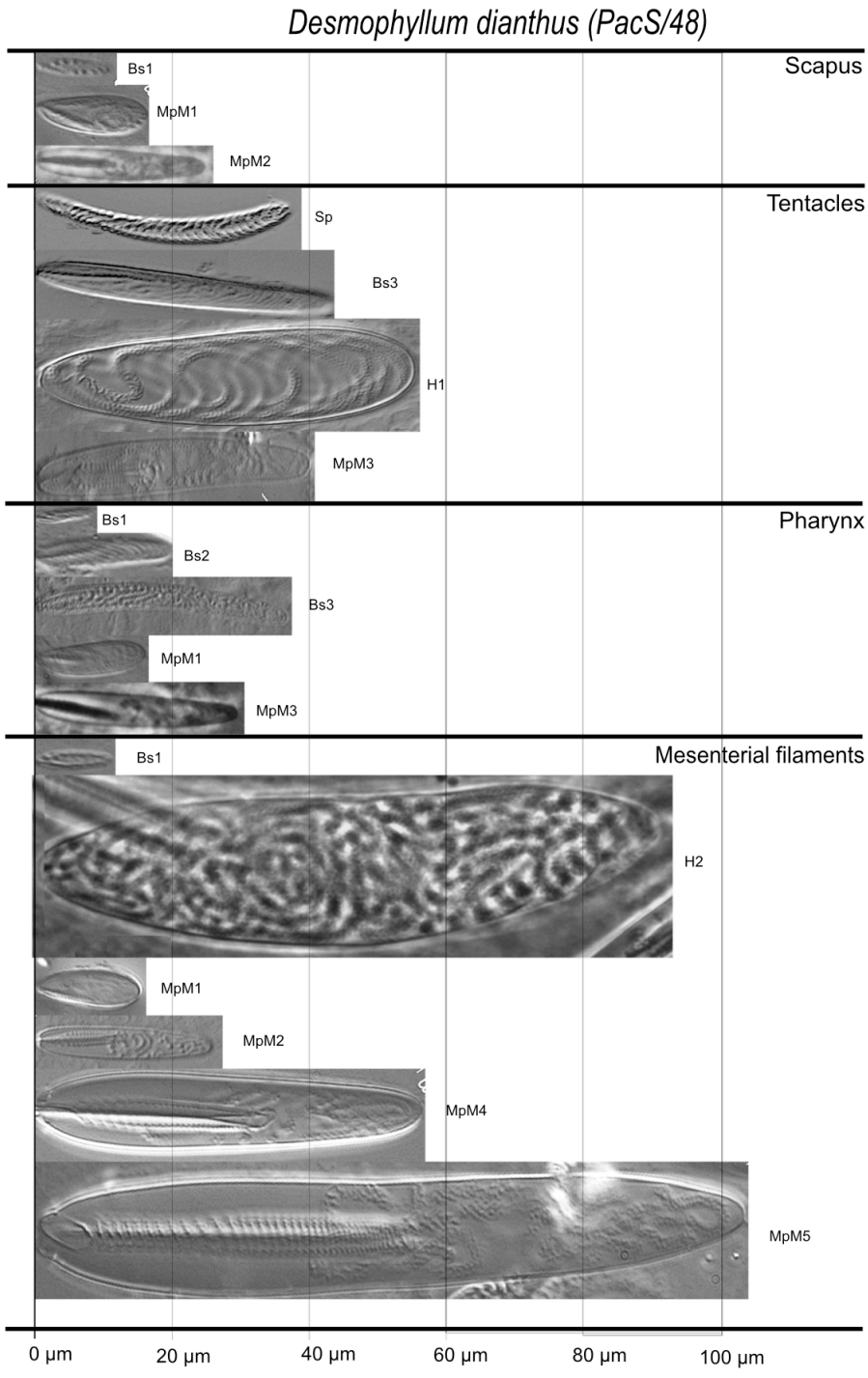


Figure 2.15. Cnidocysts of *Desmophyllum dianthus* from the South Pacific Ocean (PacS).

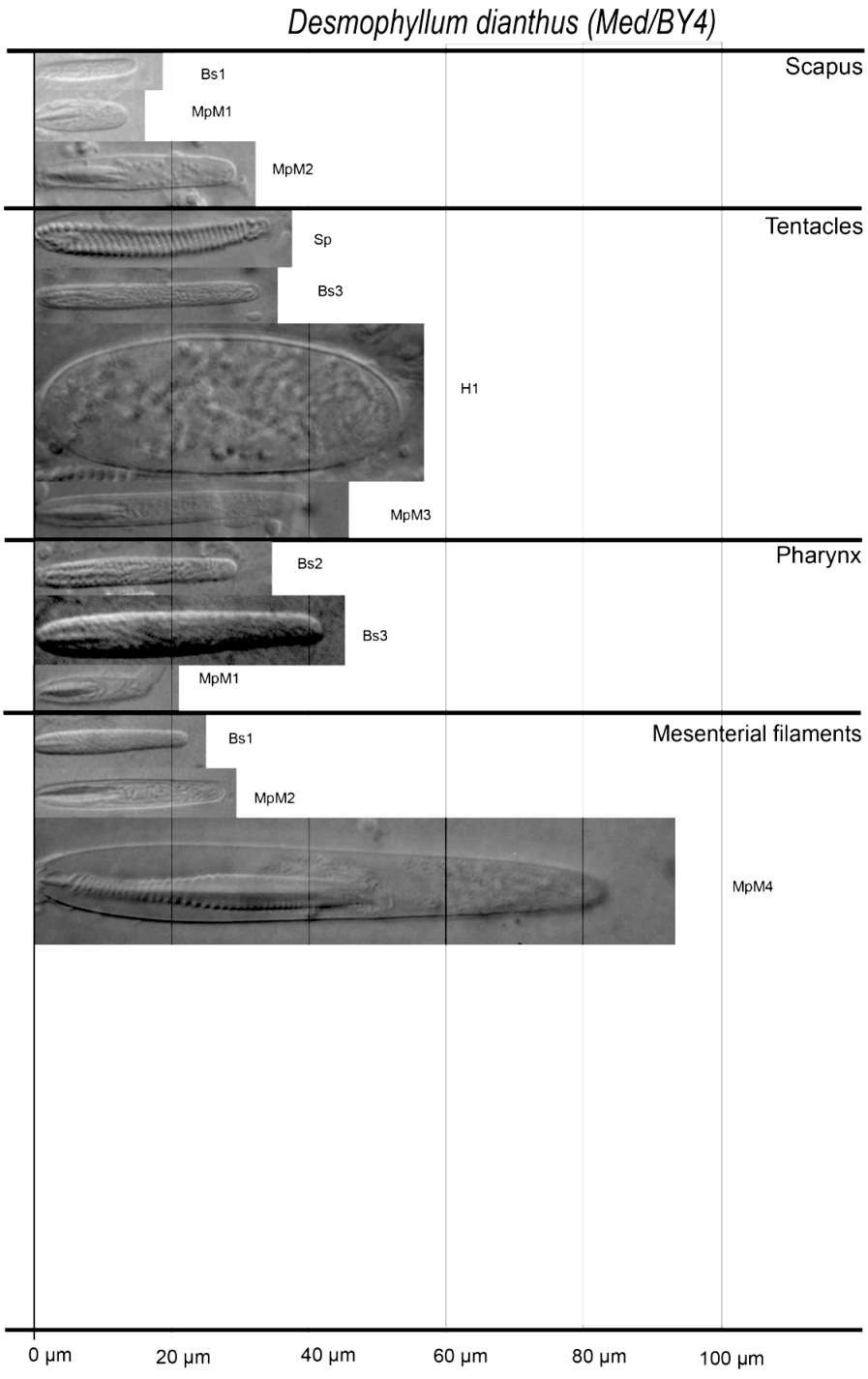


Figure 2.16. Cnidocysts of *Desmophyllum dianthus* from the Mediterranean Sea (Med

Intraspecific relationships among provinces studied here showed 60% of similarity between two distinct groups: one represented by Med/BY4 province and the other including all the leftover provinces (AtlN/BY4, AtlS/BY10, PacN/BY12, and PacS/48), (Figure 2.17). The latter group presented two additional groups of provinces: AtlS/BY10-PacS/48 and AtlN/BY4-PacN/BY12 with over 85% similarity at interindividual level. Other scleractinians (Mussiidae, Dendrophyllidae) and corallimorpharians included in the analysis were widely differentiated, with less than 30% of similarity.

The parsimony analysis (fast optimization method, minimum number of steps) on the presence-absence matrix based on 55 characters (cnidae tissue) (Table 2.21) resulted in a single tree (Figure 2.18). In the obtained tree, clade including *D. dianthus* species was no highly supported. With respect to interspecific relationships, the examined species of the family Caryophylliidae showed more affinity with species belonging to the family Dendrophylliidae than to Mussidae. There were three synapomorphies which defined the relationship between families Caryophylliidae and Dendrophylliidae (according to the sampled species): presence of Bs2S, absence of Bs3T, and absence of Bs1P (encoded as 2-1, 15-0, and 24-0 respectively). Regarding the difference within this clade, *D. dianthus* showed a series of autapomorphic (absence of H1S, presence of MpM3T, H2P, and MpM1MF; encoded as 4-0, 23-1, 29-1, and 43-1 respectively), and homoplastic (presence of MpM1S, H1S, H2S, H1P, MpM1P, Bs4MF, H2MF, H3MF, and H5MF; encoded as 8-1, 17-1, 18-1, 28-1, 31-1, 37-1, 39-1, 40-1, and 42-1 respectively) characters.

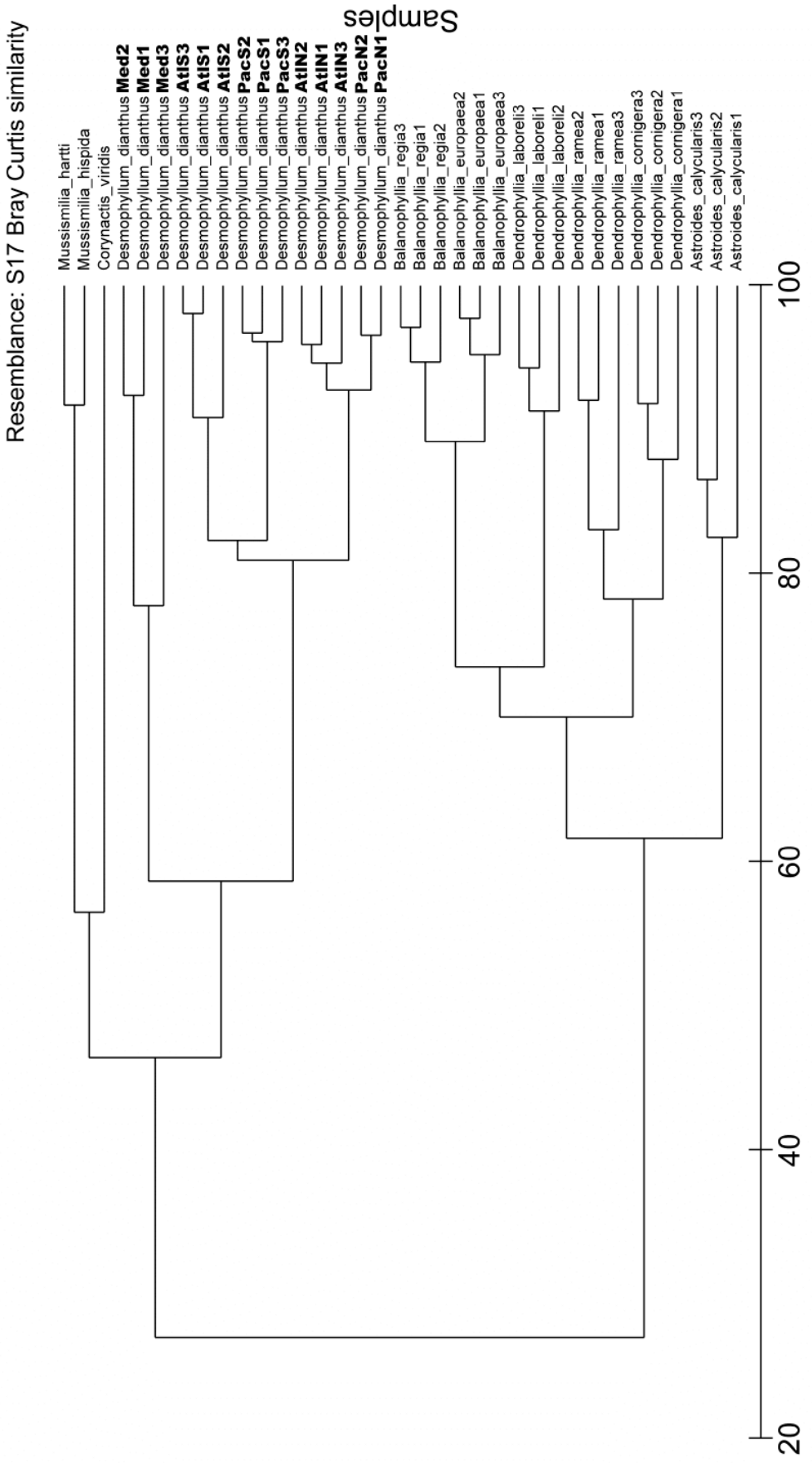


Figure 2.17. Clustering of intra- and inter-specific similarity based on the mean of cnidom.

Table 2.21. Matrix of 55 characters (presence/absence data) from all the tissues for each species.

	0	1	2	3	4	5	6	7	8	9	10	11
	SCAPUS											
Species	**SpS**	**Bs1S**	**Bs2S**	**Bs3S**	**H1S**	**H2S**	**H3S**	**H5S**	**MpM1S**	**MpM2S**	**MpM4S**	**MpM5S**
Actinauge richardi	0	1	0	0	0	0	0	0	0	0	0	0
Actinostola callosa	0	1	0	0	0	0	0	0	0	0	0	0
Bolocera tuediae	0	1	0	1	0	0	0	0	0	0	0	0
Corynactis viridis	1	1	0	1	0	0	1	0	1	0	1	0
Mussismilia hispida	1	0	0	0	0	0	0	0	1	0	0	0
Mussismilia hartti	1	0	0	0	0	0	0	0	1	0	0	0
Mussismilia braziliensis	1	0	0	0	0	0	0	0	1	0	0	0
Scolymia wellsi	1	0	0	0	0	0	0	0	1	0	0	0
Dendrophyllia cornigera	0	1	0	0	1	0	0	1	0	1	0	1
Dendrophyllia laboreli	0	0	0	1	1	0	0	1	1	1	0	0
Dendrophyllia ramea	0	1	0	0	1	1	0	1	1	1	0	0
Astroides calycularis	0	1	0	0	1	0	0	1	1	0	0	0
Balanophyllia europeae	0	1	0	0	1	0	0	1	1	0	0	0
Balanophyllia regia	0	0	0	0	1	0	0	1	0	0	0	0
Desmophyllum dianthus	0	1	1	0	0	0	0	0	1	1	0	0

	12	13	14	15	16	17	18	19	20	21	22
	TENTACLES										
Species	**SpT**	**Bs1T**	**Bs2T**	**Bs3T**	**Bs4T**	**H1T**	**H2T**	**H3T**	**H4T**	**MpM1T**	**MpM3T**
Actinauge richardi	1	1	1	0	0	0	0	0	0	0	0
Actinostola callosa	1	0	1	0	1	0	0	0	0	0	0
Bolocera tuediae	1	0	0	0	1	0	0	0	0	0	0
Corynactis viridis	1	0	0	1	1	0	0	0	1	1	1
Mussismilia hispida	1	0	1	0	0	0	0	0	0	0	1
Mussismilia hartti	1	0	1	0	0	0	0	0	0	0	1
Mussismilia braziliensis	1	0	1	0	0	0	0	0	0	0	1
Scolymia wellsi	1	0	1	0	0	0	0	0	0	0	1
Dendrophyllia cornigera	1	1	0	0	0	1	1	0	0	0	1
Dendrophyllia laboreli	1	0	0	1	0	0	1	0	0	0	1
Dendrophyllia ramea	1	0	0	0	0	0	1	0	0	0	1
Astroides calycularis	1	0	0	1	0	0	1	0	0	0	1
Balanophyllia europeae	1	0	1	1	0	0	1	0	0	0	1
Balanophyllia regia	1	0	1	1	0	0	1	0	0	0	1
Desmophyllum dianthus	1	1	0	1	0	0	0	1	0	0	1

	23	24	25	26	27	28	29	30	31
	PHARYNX								
Species	**Bs1P**	**Bs2P**	**Bs4P**	**H1P**	**H2P**	**H3P**	**MpM1P**	**MpM2P**	**MpM3P**
Actinauge richardi	1	0	0	0	0	0	1	0	0
Actinostola callosa	0	1	0	0	0	0	1	0	0
Bolocera tuediae	0	0	1	0	0	0	1	0	0
Corynactis viridis	1	0	0	0	0	1	0	0	0
Mussismilia hispida	0	1	0	0	1	0	0	0	0
Mussismilia hartti	0	1	0	0	0	1	0	0	0
Mussismilia braziliensis	0	1	0	0	0	1	0	0	0
Scolymia wellsi	0	1	0	0	0	1	0	0	0
Dendrophyllia cornigera	1	1	0	1	1	0	0	0	0
Dendrophyllia laboreli	0	1	0	1	1	0	0	1	0
Dendrophyllia ramea	1	1	0	1	1	0	0	0	0
Astroides calycularis	0	1	0	0	1	0	1	0	0
Balanophyllia europeae	0	1	0	0	1	0	0	0	0
Balanophyllia regia	0	1	0	0	1	0	0	0	0
Desmophyllum dianthus	1	1	1	0	0	0	1	0	1

Table 2.21 (continued). Matrix of 55 characters (presence/absence data) from all the tissues for each species.

	32	33	34	35	36	37	38	39	40	41	42	43	44	45
	MESENTERIAL FILAMENTS													
Species	**SMpM**	**Bs1M**	**Bs2M**	**Bs4M**	**H1M**	**H2M**	**H3M**	**H4M**	**H5M**	**MpM1M**	**MpM2M**	**MpM3M**	**MpM4M**	**MpM5M**
Actinauge richardi	0	1	0	0	0	0	0	0	0	1	0	0	0	0
Actinostola callosa	0	1	0	0	0	0	0	0	0	1	0	0	0	0
Bolocera tuediae	0	0	1	1	0	0	0	0	0	0	1	0	0	0
Corynactis viridis	0	1	0	0	0	0	1	1	0	1	0	0	0	0
Mussismilia hispida	1	1	0	0	1	1	1	0	0	1	1	1	0	0
Mussismilia hartti	1	1	0	0	1	1	1	0	0	1	1	1	0	0
Mussismilia braziliensis	1	1	0	0	1	1	1	0	0	1	1	1	0	0
Scolymia wellsi	1	1	0	0	1	0	1	0	1	1	0	1	1	0
Dendrophyllia cornigera	0	1	0	0	1	1	1	0	0	0	1	0	0	0
Dendrophyllia laboreli	0	0	0	0	1	1	0	0	0	0	1	0	0	0
Dendrophyllia ramea	0	1	0	0	0	1	0	0	0	0	1	0	0	0
Astroides calycularis	0	0	0	0	1	0	0	0	0	0	1	0	0	0
Balanophyllia europeae	0	1	1	0	1	1	0	0	0	0	1	0	0	0
Balanophyllia regia	0	1	1	0	1	1	0	0	0	0	1	0	0	0
Desmophyllum dianthus	0	0	1	0	0	0	0	1	1	0	1	1	1	1

	46	47	48	49	50	51	52	53	54
	COLUMELLA						ACONTIA	DISCO PEDAL	
Species	**Bs1C**	**H1C**	**H2C**	**H4C**	**MpM1C**	**MpM2C**	**Bs3A**	**Bs1 PD**	**PPD**
Actinauge richardi	-	-	-	-	-	-	1	1	1
Actinostola callosa	-	-	-	-	-	-	-	0	1
Bolocera tuediae	-	-	-	-	-	-	-	0	1
Corynactis viridis	-	-	-	-	-	-	-	-	-
Mussismilia hispida	-	-	-	-	-	-	-	-	-
Mussismilia hartti	-	-	-	-	-	-	-	-	-
Mussismilia braziliensis	-	-	-	-	-	-	-	-	-
Scolymia wellsi	-	-	-	-	-	-	-	-	-
Dendrophyllia cornigera	1	1	0	1	0	0	-	-	-
Dendrophyllia laboreli	1	1	1	1	0	1	-	-	-
Dendrophyllia ramea	1	1	1	1	0	1	-	-	-
Astroides calycularis	1	1	0	1	1	0	-	-	-
Balanophyllia europeae	1	1	0	1	0	0	-	-	-
Balanophyllia regia	0	1	0	1	0	0	-	-	-
Desmophyllum dianthus	-	-	-	-	-	-	-	-	-

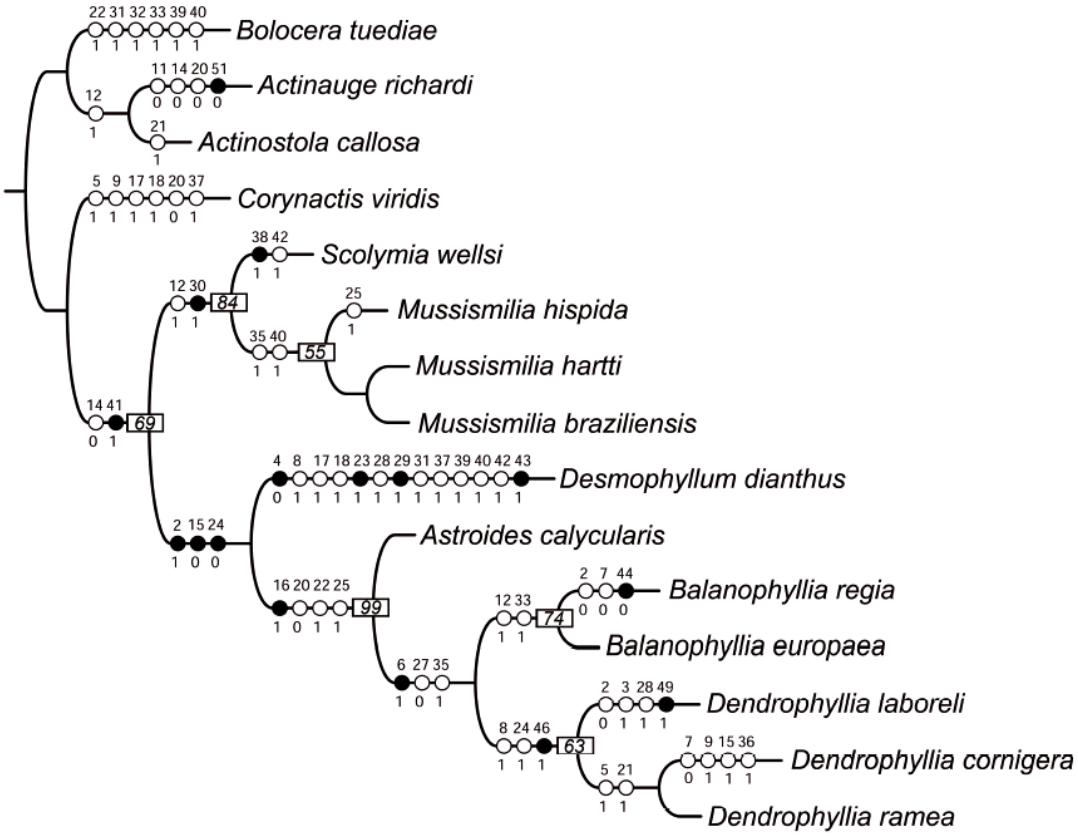


Figure 2.18. Phylogenetic hypothesis based on the presence/absence of cnidocysts in all tissues. Support lower than 50 are not represented in the tree.

The CDA indicated that H2T and Bs2P were the most contributing variables (among all shared cnidocysts) to the differences among groups (scleractinian species). The coefficients of the Fisher linear discriminant function for each localities are shown in the Tables 2.22, 2.23 and 2.24. The plot using the first two unstandardized canonical discriminant function coefficients, showed the intraspecific variation of the individuals and delimitations of the groups (Figure 2.19). Despite dendrophyllids species partially overlap due to the relative high intraspecific variability, *Dendrophyllia ramea*, *D. cornigera* and the caryophylliid *Desmophyllum dianthus* species are clearly defined.

Table 2.22. Matrix structure of CDA using cnidae characters and species. Combined intra-group correlations between discriminating variables and canonical discriminant functions typified. Variables ordered by size of correlation with function.* Largest absolute correlation between each variable and any discriminant function. (b) This variable is not used in the analysis.

	Function				
	1	2	3	4	5
H2T	,784*	0,184	-0,151	-0,457	0,347
Bs2P	-0,159	,907*	-0,243	-0,157	0,263
MpM2M	0,203	0,510	,824*	0,094	0,099
SpT	0,328	0,179	-0,182	,731*	0,541
MpM3T	0,533	0,383	-0,198	0,258	-,681*

Table 2.23. Coefficients of classification of the Fisher's linear discriminant functions for each species.

	Species						
	Dendrophyllia cornigera	*Dendrophyllia laboreli*	*Dendrophyllia ramea*	*Astroides calycularis*	*Balanophyllia europaea*	*Balanophyllia regia*	*Desmophylum dianthus*
SpT	0,380	0,204	0,364	0,274	0,227	0,280	0,413
H2T	1,968	1,266	1,631	1,209	1,368	1,339	2,178
MpM3T	1,888	1,389	1,708	1,399	1,396	1,262	1,710
Bs2P	1,509	1,743	1,910	1,484	1,573	1,217	0,544
MpM2M	1,698	1,406	0,870	1,015	1,196	1,114	1,203
(Constant)	-167,981	-104,411	-128,040	-87,021	-97,984	-81,988	-129,291

Table 2.24. Result of classification for each species. Correctly classified 78.6% of original grouped cases, and 77.8% of the grouped cases validated by cross-validation. Cross-validation applies only to cases of analysis, and each case is classified by the functions derived.(b)

			Predicted ownership group							
		Species	*Dendrophyllia cornigera*	*Dendrophyllia laboreli*	*Dendrophyllia ramea*	*Astroides calycularis*	*Balanophyllia europaea*	*Balanophyllia regia*	*Desmophylum dianthus*	Total
Original	Inventory	*D. cornigera*	126	1	5	0	0	0	3	135
		D. laboreli	6	88	1	0	29	11	0	135
		D. ramea	1	1	127	0	4	2	0	135
		A.calycularis	0	7	0	12	15	16	0	50
		B. europaea	0	22	2	1	85	24	1	135
		B. regia	0	0	0	1	28	106	0	135
		D. dianthus	8	0	4	0	0	3	178	193
	%	*D. cornigera*	93,3	0,7	3,7	0,0	0,0	0,0	2,2	100,0
		D. laboreli	4,4	65,2	0,7	0,0	21,5	8,1	0,0	100,0
		D. ramea	0,7	0,7	94,1	0,0	3,0	1,5	0,0	100,0
		A.calycularis	0,0	14,0	0,0	24,0	30,0	32,0	0,0	100,0
		B. europaea	0,0	16,3	1,5	0,7	63,0	17,8	0,7	100,0
		B. regia	0,0	0,0	0,0	0,7	20,7	78,5	0,0	100,0
		D. dianthus	4,1	0,0	2,1	0,0	0,0	1,6	92,2	100,0
Crossed validation[b]	Inventory	*D. cornigera*	125	1	6	0	0	0	3	135
		D. laboreli	7	86	1	0	30	11	0	135
		D. ramea	1	1	127	0	4	2	0	135
		A.calycularis	0	7	0	12	15	16	0	50
		B. europaea	0	24	2	1	82	25	1	135
		B. regia	0	0	0	1	28	106	0	135
		D. dianthus	9	0	4	0	0	4	176	193
	%	*D. cornigera*	92,6	0,7	4,4	0,0	0,0	0,0	2,2	100,0
		D. laboreli	5,2	63,7	0,7	0,0	22,2	8,1	0,0	100,0
		D. ramea	0,7	0,7	94,1	0,0	3,0	1,5	0,0	100,0
		A.calycularis	0,0	14,0	0,0	24,0	30,0	32,0	0,0	100,0
		B. europaea	0,0	17,8	1,5	0,7	60,7	18,5	0,7	100,0
		B. regia	0,0	0,0	0,0	0,7	20,7	78,5	0,0	100,0
		D. dianthus	4,7	0,0	2,1	0,0	0,0	2,1	91,2	100,0

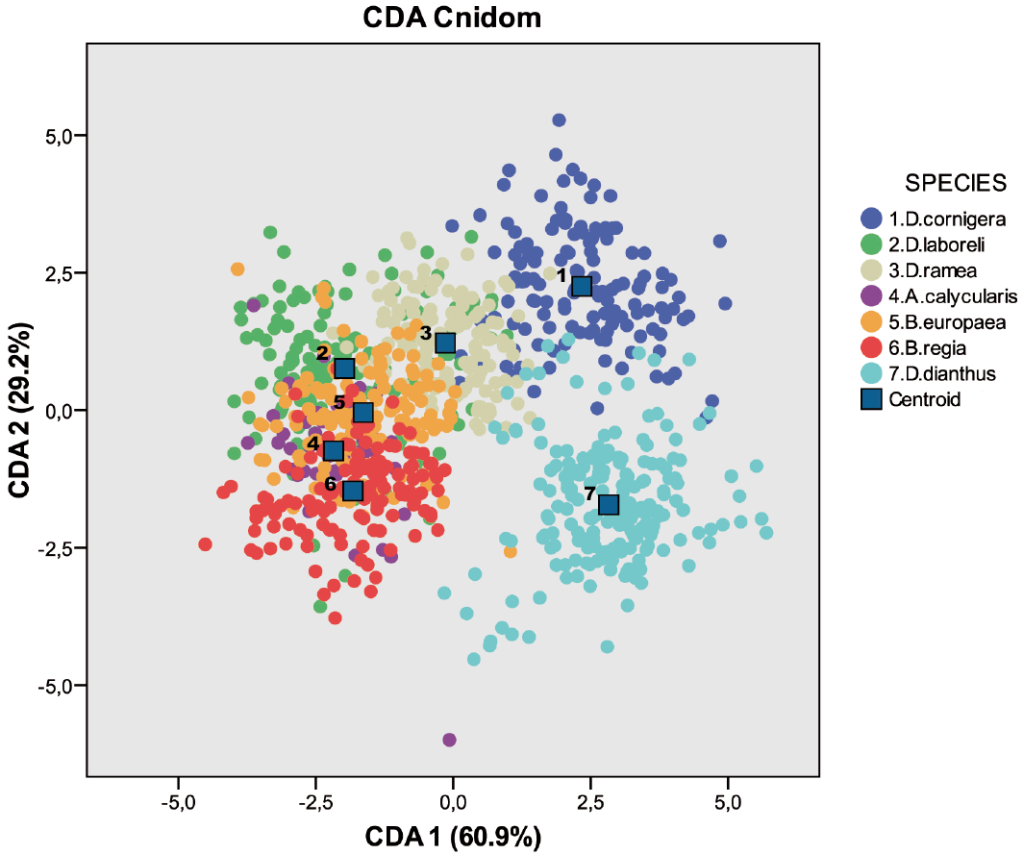


Figure 2.19. Plots of the first two functions from discriminant analyses of cnidom data in different species.

The CDA indicated that MpM4MF/MpM1S and Bs1S were the most contributing variables (among all shared cnidocysts) to the differences among groups (provinces). The analysis reached full discriminatory capacity (100%) with eight cnidocysts categories (from the eleven ones chosen *a priori*): Bs1S, MpM1S, H1T, MpM3T, Bs2P, Bs3Pm, MpM1P, and Bs1MF. Results showed that MpM1S and Bs1MF were the most contributing variables to differentiate among the five groups (provinces). The coefficients of the Fisher linear discriminant function for each localities are shown in Tables 2.25, 2.26 and 2.27. The plot using the first two unstandardized canonical discriminant function coefficients, showed the intraspecific variation of the individuals and delimitations of the groups (Figure 2.20). Some groups (localities) partially overlapped due to the relative high intraspecific variability, while other localities, such as AtlS/BY10 and Med/BY4, were quite clearly defined. The classification, evaluated through the Jack-knife validation for five categories among the dependent variable (localities), showed that more than 82.9 % of the cases were correctly classified. For

PacN/BY12 the model classified correctly 67.5 % of the cases, PacS/48 83.3 %, AtlN/BY4 63.3 %, AtlS/BY10 98.3 %, and for Med/BY4 96.76 % (Table 12a-c).

Table 2.25. Matrix structure of CDA using cnidae characters and samples classified by locality**.** Combined intra-group correlations between discriminating variables and canonical discriminant functions typified. Variables ordered by size of correlation with function.* Largest absolute correlation between each variable and any discriminant function. (b) This variable is not used in the analysis.

	Function			
	1	2	3	4
MpM4MF	,845*	-,299	-,013	-,319
Bs1MF	,287*	,027	-,075	-,185
Bs3P	,228*	,032	-,034	-,148
MpM1S	,156	,641*	,149	,149
MpM1P[b]	,024	-,099*	-,007	-,078
Bs1S	-,081	-,385	,711*	,226
MpM3T	,073	,381	,627*	-,389
H1T	,177	,112	,034	,724*
Bs2P	,147	-,126	,029	,503*
MpM2MF[b]	,096	,079	,031	-,101*
SpT[b]	-,054	,087	,062	-,092*

Table 2.26. Coefficients of classification of the Fisher's linear discriminant functions for each locality.

	Area/Province				
	PacN/BY12	PacS/BY8	AtlN/BY4	AtlS/BY10	Med/BY4
Bs1S	,801	,651	,895	,511	1,149
MpM1S	5,620	5,561	6,085	6,296	4,783
H1T	5,033	5,131	5,510	5,617	5,160
MpM3T	1,569	1,321	1,511	1,453	1,225
Bs2P	,821	,931	1,106	1,164	1,151
Bs3P	1,682	1,619	1,722	1,902	1,710
Bs1MF	,756	,774	,819	1,016	,700
MpM4MF	2,665	2,401	2,859	3,330	3,061
(Constant)	-350,806	-325,669	-403,494	-453,426	-368,822

Table 2.27. Result of classification for each locality. Correctly classified 83.9% of original grouped cases, and 82.9% of the grouped cases validated by cross-validation. Cross-validation applies only to cases of analysis, and each case is classified by the functions derived.(b)

Code Area/Province			Predicted ownership group					Total
			PacN/BY12	PacS/BY8	AtlN/BY4	AtlS/BY10	Med/BY4	
Original	Inventory	PacN/BY12	27	6	6	0	1	40
		PacS/BY8	6	51	3	0	0	60
		AtlN/BY4	4	7	39	8	2	60
		AtlS/BY10	0	0	0	60	0	60
		Med/BY4	1	1	0	0	58	60
	%	PacN/BY12	67,5	15,0	15,0	0,0	2,5	100,0
		PacS/BY8	10,0	85,0	5,0	0,0	0,0	100,0
		AtlN/BY4	6,7	11,7	65,0	13,3	3,3	100,0
		AtlS/BY10	0,0	0,0	0,0	100,0	0,0	100,0
		Med/BY4	1,7	1,7	0,0	0,0	96,7	100,0
Crossed validation[b]	Inventory	PacN/BY12	27	6	6	0	1	40
		PacS/BY8	6	50	3	0	1	60
		AtlN/BY4	5	7	38	8	2	60
		AtlS/BY10	0	0	1	59	0	60
		Med/BY4	1	1	0	0	58	60
	%	PacN/BY12	67,5	15,0	15,0	0,0	2,5	100,0
		PacS/BY8	10,0	83,3	5,0	0,0	1,7	100,0
		AtlN/BY4	8,3	11,7	63,3	13,3	3,3	100,0
		AtlS/BY10	0,0	0,0	1,7	98,3	0,0	100,0
		Med/BY4	1,7	1,7	0,0	0,0	96,7	100,0

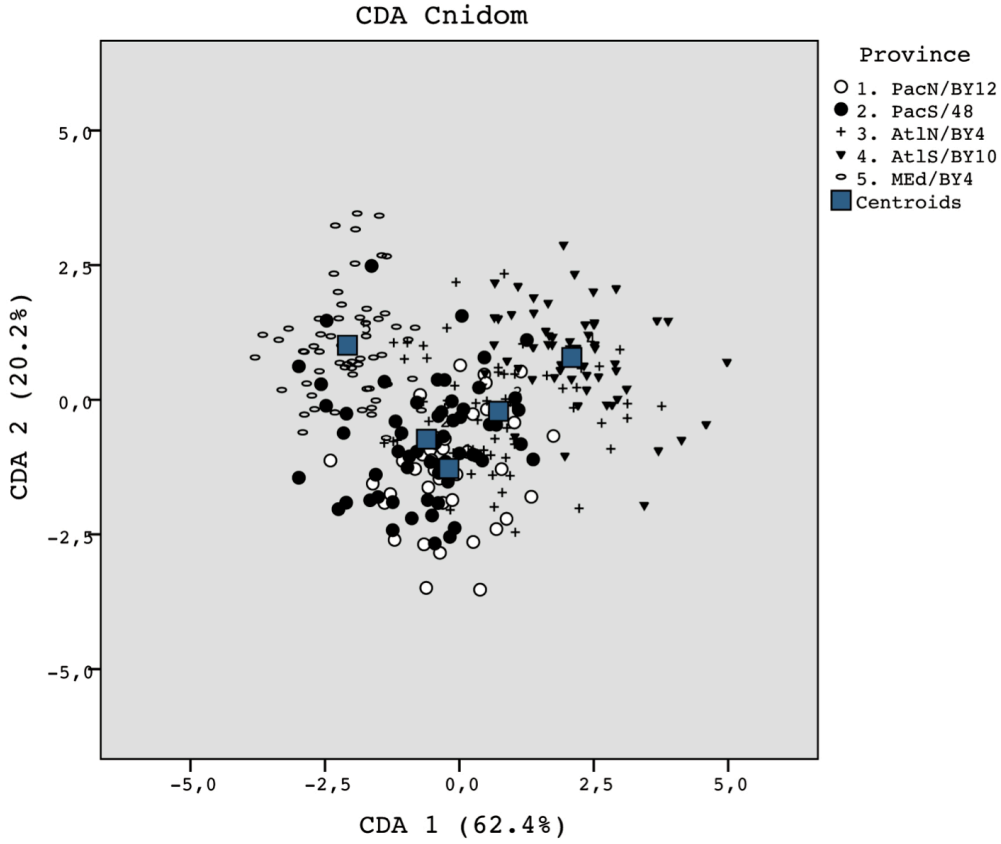


Figure 2.20. Plots of the first two functions from discriminant analyses of cnidom data in *Desmophyllum dianthus* individuals, classified by marine province.

Discussion

Skeletal analysis: morphometry of macrocharacters in solitary corals

Previous studies on the morphological variation of *D. dianthus* were conducted at interpopulation and intrapopulation level, and geographic and environmental conditions were taken into account during the analyses (Miller *et al.* 2011; Fillinger and Richter 2013).

In concordance with previous results showed in Miller *et al.* (2011), geographical pattern of morphological differentiation were found among *D. dianthus* populations; however such a pattern was lost when a large number of individuals were examined to measure variation through a wide ecological and geographic range. The analyses of macromorphological features in this study showed unresolved classification of individuals by potential morphotypes, and the morphometric characters did not even detect differences among examined biogeographic areas. These results suggested that no morphological divergence is occurring between corals in the 13 biogeographic provinces analysed and that the likely significant differences in the skeletal morphology of *D. dianthus* could be included to the high degree of phenotypic variability that characterize the species. Indeed, as Zibrowius (1983) remarked, it exhibits considerable variation in spite of its rather simple structure (absence of pali and reduced columella on only very young stages), and the shape of the corallum is largely conditioned by the substrate available for the settling larva. In contrast to reef-building zooxanthellate corals, where the ecomorphs concept (combination of high morphological and ecological diversity) is applied as an intraspecific taxonomic approach (Veron and Pichon 1976), high levels of intraspecific variation occurring in ahermatypic corals must be taken into account in species definition (Zibrowius 1983).

Considering results from this study and those previous in literature (see Miller *et al.* 2011; Addamo *et al.* 2012; Fillinger and Richter 2013), *D. dianthus* presented two different distribution and variation patterns: 1) very large geographic scale and little variation; 2) small geographical domain and high variability. The former pattern could assume that species occupying a large geographic area while showing little variation, could be regarded as an old and stable species, in contrast to the highly variable species in the same area which could be regarded as young and diversified (Wijsman-Best

1980). However it is possible that the degree of variation within the species is not closely related to the age of the genus from an evolutionary viewpoint, but it has to do with the extent of genotypic polymorphism within the species (Borel Best *et al.* 1983). The latter pattern, on the other hand, could assume that micro-environmental differences could have important impacts that are best exploited by a large phenotypic plasticity response (Bradshaw 1965). This "silent intraspecific variation" may also be visible at molecular level. Previous genetics analyses performed with nuclear (internal transcribed spacer regions, ITS) and mitochondrial (large ribosomal subunit 16S) genes showed low genetic differentiation and the occurrence of shared haplotypes between Mediterranean Sea and South Pacific populations of *D. dianthus* (Addamo *et al.* 2012).

Skeletal analysis: 3D coordinates of landmarks in solitary corals

The process of choosing landmarks is one of the most important parts of geometric morphometric analyses. The landmarks must be used to represent the shape of the object under study, and to be informative in regard to the specific aspect or question considered. Three dimensional morphometric methods are widely used in paleontology - especially in anthropology - (Bastir *et al.* 2011,) and is going to be more common among coral taxonomists who used it for colonial corals (Budd *et al.* 1994; Carlon and Budd 2002a; Grass Darrell 2009; Kongjandtre *et al.* 2012). This study provided an opportunity to explore the utility of 3D geometric morphometric methods in solitary corals. It represented a first attempt in performing morphometric analyses on solitary coral with landmarks method. Due to the nature of the landmark methods employed, usually only relatively complete specimens could be used, and two main challenges have been faced: firstly, lack of type 1 and type 2 landmarks along corallum wall, excluding a set of elements eventually informative for representing shape; secondly, although landmarks could be found along costosepta, excluding all incomplete or deformed specimens from the study (considered as an ideal practice from a morphological standpoint) resulted in affecting sample size too small in order to perform rigorous statistical analyses. The alternative for overcoming this problem could be to increase notably the amount of specimens. However there are problems associated with this. On one hand, the depth where the species is usually living has not allowed increasing the number of samples as preferred; on the other hand, the septa (one of the most characteristic corallum feature of *D. dianthus*) are easily damaged by techniques

used during sampling. Another alternative could be to exclude or decrease the numbers of landmarks defined in this study, but it eventually means to exclude or decrease informative characters. Obtained results showed a slight, but no significant, structure of *D. dianthus* populations throughout the oceans and marine regions. Such a structure is similar to the pattern found in macromorphology and cnidocyst outcomes, leading to consider landmarks as potentially informative characters at intraspecific level, and they may be the right alternative to reach accurate and reliable conclusions. However, further studies should be performed, applying a larger number of specimens, in order to confirm that such pattern could not be sample size-dependent.

Tissue analysis: characterization of the cnidom at intraspecific level

Even thought the intraspecific variability is lower than interspecific variability at geographical scale (Terrón-Sigler and López-González 2005; Martínez-Baraldés *et al.* 2014), it may be expressed at ontogenetic or ecological level (Williams 1998). Diversity and distribution of cnidocysts showed qualitative and quantitative differences among examined biogeographic units. Mediterranean Sea, Subantarctic and Chilean Fjords (BY4-MED; BY10 and 48, respectively) were the biogeographic units mostly discriminated by cnidocysts. These differentiations may reflect the environmental heterogeneity of the analysed provinces, corresponding to locations where oceanographic fronts or transitions in species and/or other environmental variables are known to occur (Watling *et al.* 2013).

The cnidae diversity could retain sufficient information at ecological level to group together examined individuals throughout biogeographic areas, since the morphology of a nematocyst may depend also on its function (Schmidt 1974). Further studies on physiology and ecological function of nematocysts could also increase their value as phylogenetic informative characters in the Anthozoa.

Several authors have also highlighted the importance of nematocysts classification: (Schmidt 1974) considered nematocyst (type, size and distribution included) as important characters to reveal a clear new concept of the evolution in the Anthozoa, but he also argued that the Weill's classification system is artificial and arbitrary, and could not coincide with a natural or phylogenetic system of the Cnidaria, pointing to mislead the phylogenetic interpretation. The value of cnidae for systematics depends on re-

evaluating their diagnostic characteristics, and systematising nematocyst nomenclature may avoid confusion by using synonyms for the nematocysts already well known (Östman 2000). As applied so far, numeral assignment to the categories of a determined type of cnidocyst depends on the number of existing size-categories of that type of cnidocyst in a determined dataset. However, when the dataset includes additional species in the comparison, another intermediate category among the previous considered categories can appear, forcing the renaming of these categories in the matrix, in order to establish a consecutive sequence of size-categories for each type (see Martínez-Baraldés *et al.* 2014 for details in nomenclature methodology). Therefore, a deeper exploration of the cnidome throughout anthozoan orders, with a standardized nomenclature and quantitative methodology may increase their potential as indicators of phylogenetic relationships and ecological conditions.

Although results from this study revealed a non-structured pattern of morphology variability in *D. dianthus* populations - probably due to its peculiar characteristics - sample and scale size used to perform each analysis, cnidocysts characters and 3D landmark coordinates, resulted as potential and useful tools to investigate morphology variation at intraspecific level. Therefore, the use of combined analyses is highly suggested in morphological studies.

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CHAPTER III

Chapter adapted from:

Addamo AM, García Jiménez R, Taviani M, Machordom A (Submitted) 454-mining of microsatellites in the deep-sea cup coral *Desmophyllum dianthus* and cross-species amplifications in the order Scleractinia. *Journal of Heredity*

Desmophyllum eburneum. Charles Joseph Gravier (1920). Madreporaires provenant des Campagnes des yachts Princesse-Alice et Hirondelle II (1893-1913).

454-mining of microsatellites in the deep-sea cup coral *Desmophyllum dianthus* (Esper, 1794) and cross-species amplifications in the order Scleractinia.

Abstract

Microsatellite loci were isolated for the first time for the deep-sea coral *Desmophyllum dianthus* (Esper, 1794), using 454 GS-FLX Titanium pyrosequencing. We developed conditions for amplifying 24 markers in 10 multiplex reactions. Three to 16 alleles per locus were detected across 25 samples analysed from Santa Maria di Leuca coral province (Mediterranean Sea). For the 24 polymorphic loci, observed and expected heterozygosities ranged from 0.211 to 0.880 and 0.383 to 0.910, respectively; three loci deviated from Hardy–Weinberg equilibrium, after null allele and sequential Holm-Bonferroni corrections. These newly isolated microsatellites are very useful genetic markers that provide data for future conservation strategies. Cross-amplification of these microsatellites, tested in 46 coral species, representing 40 genera and 10 families of the phylum Cnidaria, produced informative allelic profiles for between 1 and 24 loci.

Keywords: Microsatellites, high-throughput sequencing, deep sea coral, *Desmophyllum dianthus*, population structure

Introduction

The azooxanthellate stony coral genus *Desmophyllum* Ehrenberg, 1834 (Anthozoa, Scleractinia) is included in the family Caryophylliidae based on morphological characters (Esper, 1794) and molecular analyses (Addamo *et al.* 2012; Cuif *et al.* 2003; Kerr 2005). At the present, the genus *Desmophyllum* is considered to include three nominal species, i.e. *D. dianthus* (Esper, 1794), *D. quinarium* Tenison-Wood, 1879, and *D. striatum* Cairns, 1979 (Roberts *et al.* 2009). While the latter two species present a quite restricted geographic distribution, *D. dianthus* (syn. *Desmophyllum cristagalli* Milne Edwards & Haime, 1848) is almost cosmopolitan, with the exception of Antarctic and Arctic waters. The genus *Desmophyllum* presents a considerable geological

antiquity being documented in the Mediterranean basin since the Miocene (Taviani *et al.* 2005; Vertino *et al.* 2014). In the Mediterranean Sea, *D. dianthus* is frequently associated with other cold-water coral (CWC) reef-building species, such as *Lophelia pertusa* and *Madrepora oculata*, often contributing to the 'white coral' reef framework (Angeletti *et al.* 2013; Freiwald *et al.* 2009; Taviani *et al.* 2011b). These coral buildups provide a complex three dimensional structure with several ecological niches for a large biodiversity of associated micro- and macrofauna (Rogers 1999). They also act as a refuge for prey and as a spawning and nursery area for a variety of species, including some of notable economic interest (Tursi *et al.* 2004).

Desmophyllum dianthus mostly occurs in the upper bathyal zone (common depth range of 200-2,500 m; see Roberts *et al.* 2009 and Zibrowius 1980). Deep corals are often found in fjords ecosystems, but a surface layer of dark freshwater and the deep water forced into the shallows by the narrowing of the fjord walls, allowed these corals to grow in much shallower water than usual. Thus, regard to *D. dianthus*, records at shallower depths exist for New Zealand fjords from depths of 20 m (Grange *et al.* 1981) and Chilean fjords from depths of 8 m, where *D. dianthus* is exposed to variable water chemistry gradient conditions and is found in an unusual symbiosis with the microendolithic phototrophic alga *Ostrobium quecketii* (Försterra and Häussermann 2008). The microboring phototrophic green alga *O. quecketii* lives under the tissue of the host *D. dianthus*, and protection from grazers may be of major importance for the endoliths. But *O. quecketii* requires making exchanges with the water column through the polyp tissue. The close contact with corals inevitably also includes exchange of metabolites with the host tissue. These characteristics suggest a putative facultative and mutualistic ectosymbiosis (Försterra and Häussermann 2008). Interestingly, this solitary species displays a pseudocolonial habit, as unusually large clumps of specimens could be found in Subantarctic (Cairns 1982). Chile (Försterra *et al.* 2005) and Mediterranean Sea (Cairns 1982; Taviani *et al.* 2011a) as well.

Although scientific and conservation interest regarding CWC has expanded rapidly, information on their basic life history patterns is still rather scant, partly reflecting the difficulty of cross-season sampling of cold water corals habitats, with few exceptions (e.g. *L. pertusa*, Brooke and Järnegren 2013). Therefore, our current knowledge of deep-water coral species is largely restricted to some information about ecophysiology

(e.g. Naumann *et al.* 2011), recruitment periodicity, growth and mortality rates (Adkins *et al.* 2004; Risk *et al.* 2002; Sorauf and Jell 1977; Thresher *et al.* 2011), and reproductive biology (e.g. Waller *et al.* 2005).

As one of the most common and widely distributed deep-sea corals, *D. dianthus* is a target species as model to study oceanographic-climatic variability by deciphering geochemical signals encoded within its skeletal aragonite (Adkins *et al.* 2003; Montagna *et al.* 2006; Montagna *et al.* 2005; Montagna *et al.* 2011), and CWC response to ocean acidification (Maier *et al.* 2012). However, despite its ubiquity, little is known about the ecology, biology, and reproductive patterns of this species (Thresher *et al.* 2011).

In contrast to structure-forming colonial species, which are generally gonochoristic broadcast spawners (Veron 2000), cold-water solitary scleractinians, such as *Flabellum* sp. (Mercier *et al.* 2011; Waller and Tyler 2011), *Caryophyllia* sp. (Waller *et al.* 2005), and *Fungiacyathus* sp. (Flint *et al.* 2007; Waller *et al.* 2002), have various reproductive strategies, including hermaphroditism, gonochorism, brooding, and broadcast spawning (Dahl *et al.* 2012). The spatial distribution of genetic diversity in natural populations depends on the species' mode of reproduction, and coral species often have a mixed strategy of sexual and asexual reproduction. Determining the spatial genetic structure within and among cold-water coral populations is crucial to understanding population dynamics, assessing the resilience of cold-water coral communities, and estimating genetic effects of habitat fragmentation for conservation strategies (Dahl *et al.* 2012; Morrison *et al.* 2011).

Microsatellite markers are hypervariable regions of the genome that are particularly useful for population genetics studies. These markers provide high resolution data on population structure, which may provide insight into reproductive strategies, larval dispersal, and recruitment of the species (Underwood *et al.* 2006). To gain a basic biological understanding of this species, and to provide a new tool to aid in its sustainable management, we have developed a set of microsatellite markers for *D. dianthus*. We isolated and screened microsatellites using a multiplex-enriched library with the 454 GS-FLX Titanium pyrosequencing platform. High-throughput sequencing techniques, such as pyrosequencing, are powerful tools for isolating new markers in

genomes that have not been sequenced (Martin *et al.* 2010). This procedure has been readily and successfully applied to a large variety of taxonomic groups (Malausa *et al.* 2011).

Material and Methods

Samples and DNA extractions

Live *D. dianthus* specimens were collected in the Ionian Sea from Santa Maria di Leuca coral province (Freiwald *et al.* 2009; Taviani *et al.* 2011b) during cruise CORSARO (2006), on board the RV *Urania*. Specimens here analysed were initially preserved in 80% ethanol at 4 ºC on board, prior to being stored in absolute ethanol in the laboratory.

All necessary permits were obtained for the described field studies. This study did not involve endangered or protected species listed in the IUCN Red List of Threatened Species.

A complete information for all specimens of *Desmophyllum dianthus* and species used in this study can be found in the Tables 3.1 and 3.2, respectively.

Genomic DNA (gDNA) for pyrosequencing was extracted from the mesenteric tissue of a single *D. dianthus* specimen using the QIAGEN BioSprint 15 DNA Blood Kit (Qiagen Iberia S. L., Madrid), with slight modifications, including the optional RNase treatment and an extended period of proteinase K lysis (overnight incubation at 55 °C). DNA concentration was measured using a Nanodrop 1000 (Thermo Scientific). For microsatellite characterisation, gDNA was extracted from mesenteric tissue as above. DNA concentration was quantified using the Qubit 2.0 Fluorometer and diluted to a final concentration of 2 ng/μl.

Table 3.1. Coordinates, depth and other information on specimens of *Desmophyllum dianthus*.

Codiga MNCN	*EXPEDITION*	*VESSEL*	*Province/State*	*Precise Locality*	*Country*	*Station n°*	*Range Depth*	*Lat N (start)*	*Long W (start)*	*mt (start)*	*Lat N (end)*	*Long W(end)*	*mt (end)*	*Tecnique*	*Date (dd/mm/yr)*	*Preparation*	*Insitute Collector*
DdSML_01	CORSARO	RV Urania	Ionic Sea	SantaMaria di Leuca	Italy	CR73	671-679	39°37'29"	18°39'05"	671	39°38'07"	18°40'23"	679	Agassiz trawl	02/05/06	EtOH Ass / Box	ISMAR-CNR
DdSML_02	CORSARO	RV Urania	Ionic Sea	SantaMaria di Leuca	Italy	CR73	671-679	39°37'29"	18°39'05"	671	39°38'07"	18°40'23"	679	Agassiz trawl	02/05/06	EtOH Ass / Box	ISMAR-CNR
DdSML_08	CORSARO	RV Urania	Ionic Sea	SantaMaria di Leuca	Italy	CR73	671-679	39°37'29"	18°39'05"	671	39°38'07"	18°40'23"	679	Agassiz trawl	02/05/06	EtOH Ass / Box	ISMAR-CNR
DdSML_11	CORSARO	RV Urania	Ionic Sea	SantaMaria di Leuca	Italy	CR73	671-679	39°37'29"	18°39'05"	671	39°38'07"	18°40'23"	679	Agassiz trawl	02/05/06	EtOH Ass / Box	ISMAR-CNR
DdSML_14	CORSARO	RV Urania	Ionic Sea	SantaMaria di Leuca	Italy	CR73	671-679	39°37'29"	18°39'05"	671	39°38'07"	18°40'23"	679	Agassiz trawl	02/05/06	EtOH Ass / Box	ISMAR-CNR
DdSML_15	CORSARO	RV Urania	Ionic Sea	SantaMaria di Leuca	Italy	CR73	671-679	39°37'29"	18°39'05"	671	39°38'07"	18°40'23"	679	Agassiz trawl	02/05/06	EtOH Ass / Box	ISMAR-CNR
DdSML_25	CORSARO	RV Urania	Ionic Sea	SantaMaria di Leuca	Italy	CR73	671-679	39°37'29"	18°39'05"	671	39°38'07"	18°40'23"	679	Agassiz trawl	02/05/06	EtOH Ass / Box	ISMAR-CNR
DdSML_26	CORSARO	RV Urania	Ionic Sea	SantaMaria di Leuca	Italy	CR73	671-679	39°37'29"	18°39'05"	671	39°38'07"	18°40'23"	679	Agassiz trawl	02/05/06	EtOH Ass / Box	ISMAR-CNR
DdSML_32	CORSARO	RV Urania	Ionic Sea	SantaMaria di Leuca	Italy	CR37	548-538	39°33'14"	18°13'17"	548	39°33'29"	18°13'08"	538	Epibenthic dredge	30/04/06	EtOH Ass / Box	ISMAR-CNR
DdSML_35	CORSARO	RV Urania	Ionic Sea	SantaMaria di Leuca	Italy	CR37	548-538	39°33'14"	18°13'17"	548	39°33'29"	18°13'08"	538	Epibenthic dredge	30/04/06	EtOH Ass / Box	ISMAR-CNR
DdSML_37	CORSARO	RV Urania	Ionic Sea	SantaMaria di Leuca	Italy	CR37	548-538	39°33'14"	18°13'17"	548	39°33'29"	18°13'08"	538	Epibenthic dredge	30/04/06	EtOH Ass / Box	ISMAR-CNR
DdSML_38	CORSARO	RV Urania	Ionic Sea	SantaMaria di Leuca	Italy	CR37	548-538	39°33'14"	18°13'17"	548	39°33'29"	18°13'08"	538	Epibenthic dredge	30/04/06	EtOH Ass / Box	ISMAR-CNR
DdSML_39	CORSARO	RV Urania	Ionic Sea	SantaMaria di Leuca	Italy	CR37	548-538	39°33'14"	18°13'17"	548	39°33'29"	18°13'08"	538	Epibenthic dredge	30/04/06	EtOH Ass / Box	ISMAR-CNR
DdSML_40	CORSARO	RV Urania	Ionic Sea	SantaMaria di Leuca	Italy	CR37	548-538	39°33'14"	18°13'17"	548	39°33'29"	18°13'08"	538	Epibenthic dredge	30/04/06	EtOH Ass / Box	ISMAR-CNR
DdSML_41	CORSARO	RV Urania	Ionic Sea	SantaMaria di Leuca	Italy	CR37	548-538	39°33'14"	18°13'17"	548	39°33'29"	18°13'08"	538	Epibenthic dredge	30/04/06	EtOH Ass / Box	ISMAR-CNR
DdSML_47	CORSARO	RV Urania	Ionic Sea	SantaMaria di Leuca	Italy	CR37	548-538	39°33'14"	18°13'17"	548	39°33'29"	18°13'08"	538	Epibenthic dredge	30/04/06	EtOH Ass / Box	ISMAR-CNR
DdSML_51	CORSARO	RV Urania	Ionic Sea	SantaMaria di Leuca	Italy	CR55	501-497	39°34'55.5"	18°23'21.7"	501	39°35'20.9"	18°23'39"	497	Epibenthic dredge	01/05/06	EtOH Ass / Box	ISMAR-CNR
DdSML_52	CORSARO	RV Urania	Ionic Sea	SantaMaria di Leuca	Italy	CR55	501-497	39°34'55.5"	18°23'21.7"	501	39°35'20.9"	18°23'39"	497	Epibenthic dredge	01/05/06	EtOH Ass / Box	ISMAR-CNR
DdSML_53	CORSARO	RV Urania	Ionic Sea	SantaMaria di Leuca	Italy	CR55	501-497	39°34'55.5"	18°23'21.7"	501	39°35'20.9"	18°23'39"	497	Epibenthic dredge	01/05/06	EtOH Ass / Box	ISMAR-CNR
DdSML_54	CORSARO	RV Urania	Ionic Sea	SantaMaria di Leuca	Italy	CR55	501-497	39°34'55.5"	18°23'21.7"	501	39°35'20.9"	18°23'39"	497	Epibenthic dredge	01/05/06	EtOH Ass / Box	ISMAR-CNR
DdSML_56	CORSARO	RV Urania	Ionic Sea	SantaMaria di Leuca	Italy	CR55	501-497	39°34'55.5"	18°23'21.7"	501	39°35'20.9"	18°23'39"	497	Epibenthic dredge	01/05/06	EtOH Ass / Box	ISMAR-CNR
DdSML_59	CORSARO	RV Urania	Ionic Sea	SantaMaria di Leuca	Italy	CR55	501-497	39°34'55.5"	18°23'21.7"	501	39°35'20.9"	18°23'39"	497	Epibenthic dredge	01/05/06	EtOH Ass / Box	ISMAR-CNR
DdSML_61	CORSARO	RV Urania	Ionic Sea	SantaMaria di Leuca	Italy	CR55	501-497	39°34'55.5"	18°23'21.7"	501	39°35'20.9"	18°23'39"	497	Epibenthic dredge	01/05/06	EtOH Ass / Box	ISMAR-CNR
DdSML_62	CORSARO	RV Urania	Ionic Sea	SantaMaria di Leuca	Italy	CR39	577-540	39°33'14.8"	18°13'16.3"	577	39°33'27.4"	18°13'111"	540	Epibenthic dredge	30/04/06	EtOH Ass / Box	ISMAR-CNR
DdSML_65	CORSARO	RV Urania	Ionic Sea	SantaMaria di Leuca	Italy	CR39	577-540	39°33'14.8"	18°13'16.3"	577	39°33'27.4"	18°13'111"	540	Epibenthic dredge	30/04/06	EtOH Ass / Box	ISMAR-CNR
DdSML_69	CORSARO	RV Urania	Ionic Sea	SantaMaria di Leuca	Italy	CR39	577-540	39°33'14.8"	18°13'16.3"	577	39°33'27.4"	18°13'111"	540	Epibenthic dredge	30/04/06	EtOH Ass / Box	ISMAR-CNR

Table 3.2. List of corals used for cross-amplifications. * Outgroup included (Stylasteridae).

Family	Genus	Species	Country (Province/State, Locality)	Original Code	Repository (Institute)
Caryophyllidae	*Anomocora*	*fecunda*	Portugal (Madeira Islands)	100252	USNM- NMNH
Caryophyllidae	*Aulocyathus*	*atlanticus*	Marroco	MNHN-IK-2011-2458	MNHN
Caryophyllidae	*Caryophyllia (Caryophyllia)*	*calveri*	Italy (off Santa Maria di Leuca)	AMA-282	MNCN
Caryophyllidae	*Caryophyllia (Caryophyllia)*	*huinayensis*	Chile (Patagonia, Pitipalena Fjord)	AMA-58	MNCN
Caryophyllidae	*Caryophyllia (Caryophyllia)*	*smithii*	Spain	AMA-40	MNCN
Caryophyllidae	*Ceratotrochus*	*magnaghii*	France (Marseille, Cave Rion)	98482	USNM- NMNH
Caryophyllidae	*Conotrochus*	*funicolumna*	Wallis and Futuna Islands	98722	USNM- NMNH
Caryophyllidae	*Labyrinthocyathus*	*facetus*	United States	1114920	USNM- NMNH
Caryophyllidae	*Lophelia*	*pertusa*	Italy (off Santa Maria di Leuca)	AMA-272	MNCN
Caryophyllidae	*Paraconotrochus*	*antarctica*	SubAntarctic	AMA-44	MNCN
Caryophyllidae	*Polycyathus*	*senegalensis*	United States (Texas, West Flower Garden Bank)	1026497	USNM- NMNH
Caryophyllidae	*Pourtalosmilia*	*anthophyllites*	Spain (Algeciras)	AMA-37	MNCN
Caryophyllidae	*Solenosmilia*	*variabilis*	Argentina (Patagonia)	PATA 10/08 DR13	IEO- Gijón
Caryophyllidae	*Stephanocyathus*	*moseleyanus*	Spain (Aviles Canyon)	001-V03	IEO- Santander
Caryophyllidae	*Stephanocyathus (Acinocyathus)*	*spiniger*	Philippines (Mindoro Island)	97143	USNM- NMNH
Caryophyllidae	*Stephanocyathus (Odontocyathus)*	*weberianus*	Vanuatu (Espiritu Santo Island)	98662	USNM- NMNH
Caryophyllidae	*Tethocyathus*	*endesa*	Chile (Patagonia, Pitipalena Fjord)	AMA-59	MNCN
Caryophyllidae	*Trochocyathus*	*philippinensis*	Vanuatu (Tanna Island)	98638	USNM- NMNH
Caryophyllidae	*Vaughanella*	*concinna*	Spain (Aviles Canyon)	038-DR15	IEO- Santander
Deltocyathiidae	*Deltocyathus*	*magnificus*	Indonesia	MNHN-IK-2011-2385	MNHN
Dendrophyllidae	*Balanophyllia*	*europea*	Spain (Almeria, La Isleta del Moro)	AMA-48	MNCN
Dendrophyllidae	*Balanophyllia*	*regia*	Spain (Almeria, La Isleta del Moro)	AMA-47	MNCN
Dendrophyllidae	*Cladopsammia*	*echinata*	Indonesia (Selaru Isalnd)	97628	USNM- NMNH
Dendrophyllidae	*Dendrophyllia*	*laboreli*	Spain	AMA-39	MNCN
Dendrophyllidae	*Dendrophyllia*	*ramea*	Spain (Cadiz)	AMA-45	MNCN
Dendrophyllidae	*Eguchipsammia*	*serpentina*	United States (Hawaii, Kure Island Bank)	1072335	USNM- NMNH
Dendrophyllidae	*Endopachys*	*grayi*	Somalia (off Cape Guardafui)	98983	USNM- NMNH
Dendrophyllidae	*Thecopsammia*	*socialis*	United States (NC, Cape Fear)	1114650	USNM- NMNH
Dendrophyllidae	*Tubastrea*	*micranthus*	Yemen (Gulf of Aden, Balhalf)	Y757	UNIMIB
Flabellidae	*Flabellum*	*alabastrum*	United States (Massachussetts, Georges Bank)	1008601	USNM- NMNH
Flabellidae	*Javania*	*cailleti*	Spain (Galicia Bank)	026-DR15	IEO- Santander
Flabellidae	*Monomyces*	*rubrum nobile*	New Zealand (Cape Maria Van Diemen)	94342	USNM- NMNH
Flabellidae	*Polymyces*	*wellsi*	United States (Hawaii, Pioneer Bank)	1072331	USNM- NMNH
Flabellidae	*Truncatoflabellum*	*paripavoninum*	Philippines (Mindoro Island)	97548	USNM- NMNH
Fungiacyathidae	*Fungiacyathus*	*stephanus*	Indonesia	MNHN-IK-2011-2388	MNHN
Incertae sedis	*Cladocora*	*caespitosa*	Spain (Columbretes, Puerto Tofiño)	AMA-286	MNCN
Merulinidae	*Dipsastraea*	*pallida*	Yemen (Gulf of Aden, Bir Ali)	BA119	UNIMIB
Micrabaciidae	*Leptopenus*	*discus*	United States (California, Fielberling Guyot)	93938	USNM- NMNH
Micrabaciidae	*Rhombopsammia*	*niphada*	Indonesia (Moluccas, Sera Island)	96737	USNM- NMNH
Oculinidae	*Bathelia*	*candida*	Argentina (Patagonia)	PATA 11/08 DR04	IEO- Gijón
Oculinidae	*Cyathelia*	*axilliaris*	Philippines	MNHN-IK-2011-2495	MNHN
Oculinidae	*Madrepora*	*oculata*	Italy	AMA-51	MNCN
Oculinidae	*Oculina*	*patagonica*	Spain	AMA-50	MNCN
Pocilloporadae	*Madracis*	*pharensis*	Libanon	MNHN-IK-2011-2472	MNHN
Turbinoliidae	*Tropidocyathus*	*lessoni*	Philippines	MNHN-IK-2011-2384	MNHN
Stylasteridae	*Pliobothrus**	*symmetricus**	Spain	MNHN-IK-2011-2243	MNHN

IEO Instituto Español de Oceanografía (Gijon, Spain) (Santander, Spain)
MNCN Museo Nacional de Ciencia Naturales (Madrid, Spain)
MNHN Muséum National d'Histoire Naturelle (Paris, France)
UNIMIB University of Milano-Bicocca (Milan, Italy)
USNM- NMNH National Muesum of Natural History (Washington DC, USA)

454 GS-FLX pyrosequencing

The genomic library was constructed at the Cornell Evolutionary Genetics Core Facility (EGCF, Ithaca, NY, USA). Five micrograms of gDNA were digested completely with a restriction enzyme (five-base cutter) to generate blunt-end fragments. Linkers were ligated to the digested DNA, and the resulting fragments were enriched for microsatellites by hybridization to and magnetic capture of biotinylated repeat probes (representing two unique dimers –GT and TC; five unique trimers –TTC, GTA, GTG, TCC, and GTT; and five unique tetramers –TTTC, GATA, TTAC, GATG, and TTTG).

Enriched genomic fragments captured by streptavidin-coated magnetic beads were then amplified by PCR, ligated to Roche/454 Titanium Multiplex Identifier (MID) adapters and size fractionated in an agarose gel.

Libraries with unique adapters were pooled, and sequences were generated with Roche/454 GS-FLX Titanium reagents, protocols, and hardware. MID-sorted 454 reads were trimmed of adapter sequences and assembled with SeqMan Pro (DNASTAR). Consensus files and singleton reads were exported as FASTA files and used to detect microsatellites.

Microsatellite discovery

To isolate microsatellites and design primers for population genetic analysis, we used the programs QDD2 (Meglécz *et al.* 2007) and msatcommander 0.8 (Faircloth 2008). We searched for simple sequence repeats (SSR) with a minimum of 10 perfect motif repeats for di- and trinucleotides, and at least 8 perfect motif repeats for tetra-, penta- and hexanucleotides. To design PCR primers, we used the PRIMER 3 package (Rozen and Skaletsky 2000) implemented in QDD2 and msatcommander using default settings, except for the minimum flanking distance between primer and microsatellite (set to 20 bp) and the minimum and maximum PCR product size (set to 100-400 bp).

Primer testing

A total of 94 primer pairs were selected as potential microsatellite markers. The gDNA of 23 specimens from 12 different locations in the Mediterranean Sea and Atlantic and Pacific oceans were initially used to test for successful PCR amplification by visualising amplified products on 2% agarose gels, and evaluating the scorability of genotyped loci.

As performed for the southern geoduck *Panopea abbreviata* (Molecular Ecology Resources Primer Development *et al.* 2013), PCRs were carried out in a total volume of 10 μl with 1x PCR Biotools Standard Reaction Buffer including 2 mM $MgCl_2$, 0.5 μM forward and reverse primers, 0.2 mM of each dNTP, 1.5U DNA polymerase (Biotools), and 2 ng of template DNA. PCR amplifications were performed in a Veriti^{TM} Thermal Cycler (Applied Biosystems) with a standard profile: an initial denaturing step of 94 ºC for 3 min, followed by 35 cycles of 30 s at 94 ºC, 30 s annealing at 56 ºC, and 30 s extension at 72 ºC, and a final extension of 10 min at 72 ºC. When this profile did not amplify a specific PCR product, we tested three other annealing temperatures (T_A 50, 52 or 60 ºC) with the same cycling conditions.

Of the 94 potential loci tested, 70 were excluded due to PCR failure, monomorphism, or multiple peak profiles. Twenty-four microsatellite markers produced clear electropherogram patterns. These were selected for multiplex PCR and genotyping of 25 Santa Maria di Leuca individuals to evaluate polymorphism and population genetic parameters. Five of the amplified and genotyped microsatellites were tetranucleotide repeats, while 19 were trinucleotide repeats. These were organised in 1 tetraplex, 5 triplex, and 4 duplex by Multiplex Manager 1.0 (Holloley and Geerts 2009) (Table 3.3).

Multiplex PCRs were performed in a total volume of 10 μl, which included approximately 2 ng of DNA, 1X Qiagen Multiplex PCR Master Mix (Qiagen, Hilden, Germany), and 3 mM $MgCl_2$. Tagged primer concentrations ranged from 0.5 to 0.8 mM. To facilitate genotyping, the forward primer from each primer pair was fluorescently 5' end labelled with either 6-FAM, NED, VIC, or PET, while reverse primers were pig-tailed with 5′-GTTTCTT-3′ (Brownstein *et al.* 1996). The cycling profile began with an enzyme activation step at 95 ºC for 15 min (per Qiagen Multiplex PCR kit specifications), followed by 35 cycles of 30 s at 94 ºC, 90 s annealing at 56 ºC (except for DdL24, Dd90, and Dd98, which used an annealing temperature of 60 ºC) and 60 s extension at 72 ºC, and a final extension of 30 min at 60 ºC. An Eppendorf Mastercycler gradient thermal cycler (Eppendorf AG, Hamburg, Germany) was used for all reactions.

Table 3.3. Characteristics of: locus name, repeat motif, primer sequence, annealing temperature, combination loci, and fluorescent label.

Locus	Primer sequence (5'-3')	Primer sequence (5'-3')	Repeat Motif	Size Range (bp)	TA (°C)	Multiplex	Fluorescent
DdL7	F:VIC-ACTTGTGTTTGCACCAGGG	R:GTTTCTTCAGGTCTCTGCATGCTGTC	(CAA) CAG (CAA)10	214-281	56	7	VIC
DdL13	F2:PET-TGTTAATCACATCACTAAGGTACAGC	R2:GTTTCTTSAWTTCAACTTGTACTGTTTAAATG	(GTT)15	86-122	56	10	PET
DdL16	F2:NED-ATTAGATAAACCTTGTAATGTCTGC	R2:GTTTCTTTCACAGAATGTGCAAGCTAC	(CAA)11	104-161	56	9	NED
DdL22	F2:VIC-AACATTCCATATTTGGCTTTCG	R2:GTTTCTTGCCTGCTAAATCGCCTTGTA	(ACA)13	119-168	56	10	VIC
DdL24	F:PET-GCAGACGAGGATGTGCTTG	R:GTTTCTTAATGCTGAAAGCAGCGGAC	(TGTT)10	162-212	60	4	PET
DdL34	F2:PET-CACGTGAACTGTGAAGCAGAA	R2:GTTTCTTAACGCCAGAACTGAATTAACG	(GAT)14	176-202	56	7	PET
DdL41	F:PET-ACAACGGATCTACTTTCATTCACTC	R:GTTTCTTTGATTATCGATGCAACTCATCC	(GAT)11	150-183	56	2	PET
DdL50	F:6FAM-ATCACTCGAGGGGTTCTGG	R3:GTTTCTTATTTAGGAACTCGATCACCATAGC	(TTG)11 (CTGTTG)3	194-281	56	8	FAM
DdL56	F:VIC-CGGAAAACCACATGTTGAAG	R:GTTTCTTCAGAAAACTGAAAAGGGGACAT	$(GTTT)_6$ / $(GTTT)_8$	116-148	56	1	VIC
DdL57	F:VIC-AGGGCCACAAGTTTATTAGCC	R:GTTTCTTAAAGGGCCTGTTATGCTATGAG	(TTAC)5	117-137	56	2	VIC
DdL58	F:VIC-CTTTGCCTCAGCAATGAACG	R:GTTTCTTACAGTTAGGAGCTGGTAACAATG	(TCTT)6	105-153	56	8	VIC
DdL73	F:PET-AGGCAATGCAATTCTTTGATA	R:GTTTCTTCGGAAAGAGAAAATGTGAACC	(TTTG)7	158-178	56	1	PET
DdL82	F:VIC-GGCGTAGCTCCTTTAAGCTG	R:GTTTCTTGGACGCCTTTCTTGTCTTGA	(GTT)12	116-134	56	6	VIC
DdL83	F:NED-ACTTTCCTGGCTGCTTTGAT	R:GTTTCTTGTCTCTACCCCTGCCGTACA	(TGT)8 (GGT)2 / (TGT)	230-260	56	1	NED
DdL84	F2:VIC-GACCATAACTCTATATGAGACCAAACC	R2:GTTTCTTGCCGATATTTGTATCCAATTCTACC	(CAA)6 CAG CAA	135-210	56	9	VIC
DdL86	F:6FAM-AGTCATCCTCGGCAATTTGA	R:GTTTCTTGTGACGGCATGTGGGTTAGT	(CAA)7	146-152	56	5	FAM
DdL90	F:VIC-TCCTTGAGTGAAACATCACGA	R:GTTTCTTGCCTTATGCCCCTACTGGT	(ATC)5 GTC (ATC) GTC	104-119	60	3	VIC
DdL91	F:6FAM-CGACCAAGGAAGTAGCTTGT	R:GTTTCTTGAAGGCGGAGGACCTAATAC	(GGT)9	205-223	56	1	FAM
DdL96	F:VIC-TTCCAATTCCCTTTCTTTAGCC	R:GTTTCTTCTCGTGAAATTTGGGAGCTG	(AAC)7	102-156	56	5	VIC
DdL98	F:NED-AAGCAATAACGAGAGCAACACA	R:GTTTCTTAGGGACTGGAACATGACGAA	(TCA)8	135-150	60	3	NED
DdL100	F:NED-GTGCTGCTACATGGGTGGAT	R:GTTTCTTATGTCCTGACACCATCTCGC	(TCA)9	128-140	56	6	NED
DdL102	F:NED-CAAACCCTGCCTGCAGTTAC	R:GTTTCTTAGTTCAAGAAAGGGATGATAGGAA	(TGT)6	123-152	56	7	NED
DdL107	F:NED-GGTTGCTATAGCTATGTGGATTAACAG	R:GTTTCTTGAACACGAAGGGCAAGAAGA	(TTG)7	143-179	56	8	NED
DdL109	F:VIC-GCCTGGGCTTGATGTAAAGT	R:GTTTCTTACGAGCAGATTCAAGTACGG	(TGT)6	128-140	56	4	VIC

Successfully amplified loci were also tested for cross amplification in 45 species of Scleractinia (Anthozoa) and one species of Stylasteridae (Hydrozoa). A complete table of all species can be found in Table 3.2. Fluorescently labelled PCR products were run on an ABI PRISM 3730 DNA Sequencer (Applied Biosystems), scored using the GeneScan-500 (LIZ) size standard, and analysed with the GeneMapper software (Applied Biosystems).

Characterisation of novel microsatellite markers

Tests for Hardy-Weinberg equilibrium (HWE) and the presence of linkage disequilibrium (LD) for the newly developed markers were carried out in Genepop 4.1 (Rousset 2008) using default setting parameters; P values were adjusted with sequential Holm-Bonferroni corrections. Estimates of null allele frequency, error scoring, and large allele dropout were made using Micro-Checker (Van Oosterhout *et al.* 2004). Basic parameters of genetic variability and probability of identity (PI) were calculated using GenAlEx 6.5 (Peakall and Smouse 2012). Polymorphic information content (PIC) was calculated using Cervus 3.0 (Marshall *et al.* 1998).

Results

Next generations sequencing: 454 pyrosequencing results

A total of 41,913 contigs with an average size of 334 bp (57.31% of the assembled reads) were obtained, consisting of 23,947,294 nucleotides, which accounts for approximately 5.70% of the *D. dianthus* genome, assuming a genome size of 420 Mb - estimated from the size of *Acropora digitifera* (Shinzato *et al.* 2011) - (Table 3.4). Contigs containing the microsatellites identified in this study will be deposited in GenBank.

Table 3.4. Summary characteristic data. Raw data statistic from Roche 454 Sequencing. * with perfect repeats searching in MsatCommander 0.8. Min Repeats: (Di-Tri)10; (Tetra-Penta-Hexa)8 & Size prd:100-450. **with repeat searching in QDD2. Min Repeats: 5.

Total reads	73127
Total contigs	41913
Mean contigs length	334
%SSR abundance	6,8
sequences containing SSRs	2819*/5930**
Unique sequences containing SSRs	1351
Number of sequences with primers	776*/774**
Filtered dinucleotide SSR sequences	510*/227**
Filtered trinucleotide SSR sequences	97*/210**
Filtered tetranucleotide SSR sequences	146*/126**
Filtered pentanucleotide SSR sequences	14*/9**
Filtered hexanucleotide SSR sequences	9*/3**
Trinucleotide Primer3 sequences	60*/115**
Tetranucleotide Primer3 sequences	57*/50**
Pentanucleotide Primer3 sequences	2*/2**
Hexanucleotide Primer3 sequences	2*/2**
Primer pairs tested	100
Loci amplified	26
Loci scorable	24
Amplicon length, average	250
Number of individuals	25
Mean number of alleles per locus	9.13
Mean proportion of individuals typed	0.9550
Mean expected heterozygosity	0.7560
Mean polymorphic information content (PIC)	0.7123

Genome-wide microsatellite characterisation, screening, and primer testing

Perfect microsatellite sequences were identified for marker development by screening the unique sequences for 2-6 bp repeats. In total, msatcommander and QDD2 identified 2,819 microsatellites of at least 8 repeat units and 5,930 loci of at least 5 repeat units, respectively. Of these, 43.31% were dinucleotide, 9.19% trinucleotide, 35.43% tetranucleotide, 9.68% pentanucleotide, and 2.38% hexanucleotide repeats. Primers were successfully designed for 776 microsatellites, and 94 primer pairs were chosen for testing and PCR characterisation. In total, 24 of the 94 primer pairs produced reliable PCR amplicons that were scorable (Table 3.3). Of these, the number of alleles (N_A) per locus varied from 3 to 16, with an average of 9.13. Polymorphism information content (PIC) estimates were reasonably to highly informative, as defined by (Botstein *et al.* 1980), who categorised loci with a PIC> 0.5 as highly informative, 0.5> PIC> 0.25 as

reasonably informative, and PIC< 0.25 as slightly informative (Table 3.5). Observed (H_{Obs}) and expected (H_{Exp}) heterozygosities averaged 0.614 and 0.756, respectively, whereas the fixation index F_{IS} obtained across the loci ranged from -0.008 to 0.47 (Table 3.5).

The probability of two randomly sampled corals having identical genotypes, based on the first 4 loci (DdL7, DdL13, DdL16 and DdL22), was estimated at 2.6×10^{-6}, with a cumulative probability of exclusion (PE) of 99.19%, 95.44%, and 99.97% when, respectively, the genotypes of both parents were known, when only one parent was known, and when two putative parents were excluded. In the extreme situation that all individuals were in full-sibling relationships (considering the combination of all 24 loci), the probability of identity (PI) was estimated at 2.7×10^{-10}, and no matching multilocus genotypes were found. Therefore, the 4 microsatellites panel is theoretically sufficient for individual identification of any coral in the analysed population.

Tests for linkage disequilibrium (LD) yielded one (DdL7, DdL102) significant P value (following the sequential Holm-Bonferroni correction) out of 276 pairwise comparisons; none of the remaining markers showed significant LD (P> 0.06), indicating that the loci are unlikely to be physically linked. Five markers exhibited significant deviations from HWE, notably having fewer heterozygotes than expected (Table 3.5). Null allele frequencies were calculated using the Brookfield-1 method (Brookfield 1996) and the program Micro-Checker. Loci DdL22 and DdL107 were determined to have false homozygote genotypes (Table 3.5). Others potential causes are likely for loci DdL7, DdL58, and DdL84 where the presence of null alleles could not explain the excess of homozygosis and consequent departure from HWE.

Table 3.5. Statistic summary of 24 polymorphic SSR markers in *Desmophyllum dianthus.* NA the number of alleles per locus, Number of individuals with successful amplification, HExp expected heterozygosity, HObs observed heterozygosity, FIS Fixation index or Allele frequency based correlation or inbreeding coefficient, PIC polymorphic information content, pHWE, Null freq. null allele frequency. P value from exact tests of HWE. * shows significant departure from HWE after Bonferroni correction/ Significant deviation from HWE after application of Holm Bonferroni. (Pvalue) after adjustment with Brookfield-1 based on presence of null allele by Brookfield-1 estimator of null alleles (NullEst).

Locus	N_A	N	H_{Obs}	H_{Exp}	F_{IS}	PIC	p_{HWE} (Exact test)	Brookfield 1 NullEst
DdL7*	16	24	0.542	0.910	0.4103	0.883	0.0000 (0.0016)*	**0,1849**
DdL13	8	25	0.600	0.759	0.2131	0.717	0.1182	0,0826
DdL16	14	25	0.880	0.860	-0.0233	0.828	0.9942	0
DdL22*	10	24	0.583	0.879	0.3415	0.846	0.0004 (0.0244)	0,1493
DdL24	11	24	0.625	0.865	0.2820	0.830	0.0069	0,1203
DdL34	12	25	0.880	0.816	-0.0809	0.775	0.4189	0
DdL41	14	23	0.783	0.898	0.1306	0.867	0.0025	0,0508
DdL50	13	25	0.800	0.737	-0.0872	0.708	0.9066	0
DdL56	9	25	0.840	0.781	-0.0769	0.738	0.3639	0
DdL57	6	22	0.455	0.816	0.4488	0.767	0.0030	0,1908
DdL58*	8	19	0.211	0.750	0.7247	0.691	0.0000 (0.0000)*	**0,3002**
DdL73	7	20	0.800	0.737	-0.0877	0.672	0.9768	0
DdL82	6	25	0.760	0.776	0.0215	0.722	0.1484	0,0005
DdL83	8	25	0.840	0.771	-0.0909	0.721	0.5202	0
DdL84*	16	23	0.478	0.902	0.4756	0.873	0.0000 (0.0000)*	**0,2149**
DdL86	3	25	0.480	0.411	-0.1731	0.347	0.3042	0
DdL90	6	23	0.435	0.695	0.3794	0.634	0.0140	0,1458
DdL91	6	25	0.560	0.757	0.2640	0.704	0.0290	0,1043
DdL96	8	25	0.360	0.526	0.3197	0.494	0.0321	0,1024
DdL98	5	23	0.261	0.383	0.3231	0.358	0.0923	0,0825
DdL100	5	25	0.680	0.695	0.0216	0.643	0.4914	0,0005
DdL102	10	24	0.833	0.821	-0.0155	0.786	0.3242	0
DdL107*	13	25	0.640	0.905	0.2973	0.877	0.0020 (0.0111)	0,131
DdL109	5	24	0.417	0.692	0.4034	0.616	0.0055	0,1557

Cross-species transferability

In this study, cross-amplification tests for 46 species, representing 40 genera and 10 families of Scleractinia, provided interpretable profiles for at least one locus (e.g. for the species *Eguchipsammia serpentina, Endopachys grayi, Flabellum alabastrum,* and *Oculina patagonica*), but up to 24 loci per species (*Lophelia pertusa*) (Table 3.6).

Caryophylliidae, as expected being the family of *D. dianthus*, was the family with the most successful amplifications, followed by Dendrophyllidae, with 24 and 14 loci, respectively, producing interpretable genotypes. Further detailed studies are ongoing for *L. pertusa*, given its genetic similarity with *D. dianthus*, as previously recognised by nuclear and mitochondrial marker analyses (Addamo *et al.* 2012). Scorable genotyping data were also recorded for loci L82, L83, L98, and L107 in a non-Anthozoan species, *Pliobothrus symmetricus*, which belongs to the family Stylasteridae (Order Anthoathecata, Class Hydrozoa) (Table 3.7).

Table 3.6. Markers transferability: 46 Cross-species amplifications with locus scorable indication (√).* Outgroup included (Stylasteridae).

Family	Genus	Species	DdL7	DdL13	DdL16	DdL22	DdL24	DdL34	DdL41	DdL50	DdL56	DdL57	DdL58	DdL73	DdL82	DdL83	DdL84	DdL86	DdL90	DdL91	DdL96	DdL98	DdL100	DdL102	DdL107	DdL109
Caryophyllidae	*Anomocora*	*fecunda*									√		√									√				
Caryophyllidae	*Aulocyathus*	*atlanticus*								√					√	√									√	
Caryophyllidae	*Caryophyllia (Caryophyllia)*	*calveri*	√			√				√			√		√	√									√	
Caryophyllidae	*Caryophyllia (Caryophyllia)*	*huinayensis*	√	√	√	√				√			√			√	√			√	√	√		√		√
Caryophyllidae	*Caryophyllia (Caryophyllia)*	*smithii*	√													√										
Caryophyllidae	*Ceratotrochus*	*magnaghii*					√			√									√			√				
Caryophyllidae	*Conotrochus*	*funicolumna*	√												√			√					√			
Caryophyllidae	*Labyrinthocyathus*	*facetus*								√		√														
Caryophyllidae	*Lophelia*	*pertusa*	√	√	√	√	√	√		√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
Caryophyllidae	*Paraconotrochus*	*antarctica*	√	√		√				√			√	√	√	√									√	
Caryophyllidae	*Polycyathus*	*senegalensis*	√			√				√						√			√				√			
Caryophyllidae	*Pourtalosmilia*	*anthophyllites*										√														
Caryophyllidae	*Solenosmilia*	*variabilis*	√	√						√				√	√							√				
Caryophyllidae	*Stephanocyathus*	*moseleyanus*				√			√	√		√								√		√				
Caryophyllidae	*Stephanocyathus (Acinocyathus)*	*spiniger*					√													√						
Caryophyllidae	*Stephanocyathus (Odontocyathus)*	*weberianus*													√		√				√	√				
Caryophyllidae	*Tethocyathus*	*endesa*	√							√						√	√						√			
Caryophyllidae	*Trochocyathus*	*philippinensis*															√									
Caryophyllidae	*Vaughanella*	*concinna*			√															√						
Deltocyathiidae	*Deltocyathus*	*magnificus*																			√					
Dendrophyllidae	*Balanophyllia*	*europea*	√				√						√													√
Dendrophyllidae	*Balanophyllia*	*regia*								√					√							√				
Dendrophyllidae	*Cladopsammia*	*echinata*								√							√									
Dendrophyllidae	*Dendrophyllia*	*laboreli*								√							√									√
Dendrophyllidae	*Dendrophyllia*	*ramea*			√						√									√	√			√		
Dendrophyllidae	*Eguchipsammia*	*serpentina*			√											√										
Dendrophyllidae	*Endopachys*	*grayi*							√																	
Dendrophyllidae	*Thecopsammia*	*socialis*								√					√	√										
Dendrophyllidae	*Tubastraea*	*micranthus*								√						√										
Merulinidae	*Dipsastraea*	*pallida*											√			√					√					
Flabellidae	*Flabellum*	*alabastrum*			√																					
Flabellidae	*Javania*	*cailleti*	√							√			√													
Flabellidae	*Monomyces*	*rubrum nobile*				√							√		√							√				
Flabellidae	*Polymyces*	*wellsi*			√					√										√					√	
Flabellidae	*Truncatoflabellum*	*paripavoninum*								√	√									√					√	
Fungiacyathidae	*Fungiacyathus*	*stephanus*	√																		√			√		
Micrabaciidae	*Leptopenus*	*discus*				√									√					√			√			
Micrabaciidae	*Rhombopsammia*	*niphada*				√				√			√							√		√		√		
Oculinidae	*Bathelia*	*candida*														√								√		
Oculinidae	*Cyathelia*	*axilliaris*														√							√			
Oculinidae	*Madrepora*	*oculata*	√							√					√	√						√				
Oculinidae	*Oculina*	*patagonica*				√																				
Pocilloporadae	*Madracis*	*pharensis*													√					√						
Incertae sedis	*Cladocora*	*caespitosa*										√										√			√	
Turbinoliidae	*Tropidocyathus*	*lessoni*													√			√				√				
Stylasteridae	*Pliobothrus**	*symmetricus**													√	√						√			√	

Table 3.7. Marker transferability. Number of successful cross-species amplifications per family and locus.*Outgroup included.

	Caryophylliidae	Deltocyathidae	Dendrophylliidae	Merulinidae	Flabellidae	Fungiacyathidae	Micrabaciidae	Oculinidae	Pocilloporidae	Turbinoliidae	Stylasteridae*	Tot No.Species	Tot No.Genera	Tot No.Family
DdL7	9	0	1	0	1	1	0	1	0	0	0	13	11	5
DdL13	4	0	0	0	0	0	0	0	0	0	0	4	4	1
DdL16	3	0	2	0	2	0	0	0	0	0	0	7	7	3
DdL22	6	0	0	0	0	0	0	0	0	0	0	6	5	1
DdL24	3	0	1	0	1	0	2	1	0	0	0	8	8	5
DdL34	1	0	0	0	0	0	0	0	0	0	0	1	1	1
DdL41	2	0	1	0	0	0	0	0	0	0	0	3	3	3
DdL50	10	0	4	0	3	0	1	1	0	0	0	19	18	5
DdL56	2	0	2	0	1	0	0	0	0	0	0	5	5	3
DdL57	2	0	0	0	0	0	0	0	0	0	0	2	2	1
DdL58	9	0	1	1	2	0	1	0	0	0	0	14	13	5
DdL73	1	0	0	0	0	0	0	0	0	0	0	1	1	1
DdL82	4	0	0	0	1	0	1	1	0	1	1	9	9*	6*
DdL83	7	0	2	0	0	0	0	1	1	0	1	12	11*	5*
DdL84	6	0	3	1	0	0	0	3	1	0	0	14	13	6
DdL86	7	0	3	0	1	0	0	0	0	0	0	11	11	3
DdL90	3	0	0	0	0	0	1	0	0	1	0	5	5	3
DdL91	5	0	0	0	0	0	2	0	0	0	0	7	6	2
DdL96	3	1	2	1	2	0	0	0	1	0	0	10	9	6
DdL98	7	0	2	0	0	1	1	1	0	1	1	14	13*	7*
DdL100	4	0	0	0	1	0	1	3	0	0	0	7	7	4
DdL102	3	0	1	0	1	0	2	1	0	0	0	8	8	5
DdL107	4	0	0	0	2	0	1	0	0	0	1	8	8*	4*
DdL109	4	0	2	0	0	0	0	0	0	0	0	6	5	2

Repeat distribution across species

Pattern repeat distribution in *D. dianthus* was compared with two other marine invertebrates previously analysed by the same sequencing method: a mollusc, *Panopea abbreviata* and a nemertean, *Malacobdella arrokeana* (Alfaya *et al.* 2014; Molecular Ecology Resources Primer Development *et al.* 2013). Although more sequences were obtained for *D. dianthus* (Table 3.8), *P. abbreviata* had the highest proportion of sequences containing repeats (26% compared to 6.09% and 4.21% in *D. dianthus* and *M. arrokeana*, respectively). We characterised each of these marine invertebrates by the frequency of repeat type, repeat motif, and repeat length (Table 3.8, Figure 1). *Desmophyllum dianthus* showed a relatively high frequency of penta- and hexanucleotide repeats, while *P. abbreviata* and *M. arrokeana* had higher frequencies

of tetranucleotide and trinucleotide repeats, respectively. Dinucleotide repeats accounted for approximately 40% of repeats in all three species (Table 3.8, Figure 1). Common probes were used to detect di-, tri- and tetranucleotides repeats, therefore common repeat motifs were observed among species. However, *D. dianthus* had the highest frequency of unique repeat type (67.44%) (Table 3.8).

Table 3.8. Comparative approach of repeat classes in three marine invertebrates. Genomic distribution of 2–6bp repeat motifs and classes for *Desmophyllum dianthus*, *Malacobdella arrokeana* and *Panopea abbreviata*.

Species	**Genome size (Mb)**	**Evolutionary domain**
Desmophyllum dianthus	420	animal/cnidaria
Panopea abbreviata	1344,8	animal/nemertea
Malacobdella arrokeana	1369,2	animal/mollusca

Repeat class	***D.dianthus***	***P.abbreviata***	***M.arrokeana***
DI	1221	881	337
TRI	259	327	256
TETRA	999	1004	46
PENTA	273	27	2
HEXA	67	7	3
Sequences containing repeats	2552	1878	728
TOTAL number of repeats found	2819	2246	784
Sequences analyzed for repeats	41913	7192	17273

Repeat type	***D.dianthus***	***P.abbreviata***	***M.arrokeana***	**Tot**	**Common repeat type**
Dinucleotidos	2	3	3	3	2
Trinucleotidos	7	10	4	10	3
Tetranucleotidos	21	20	12	30	7
Pentanucleotidos	16	9	2	25	
Hexanucleotidos	10	5	3	18	
Unique repeat type	29	20	8		

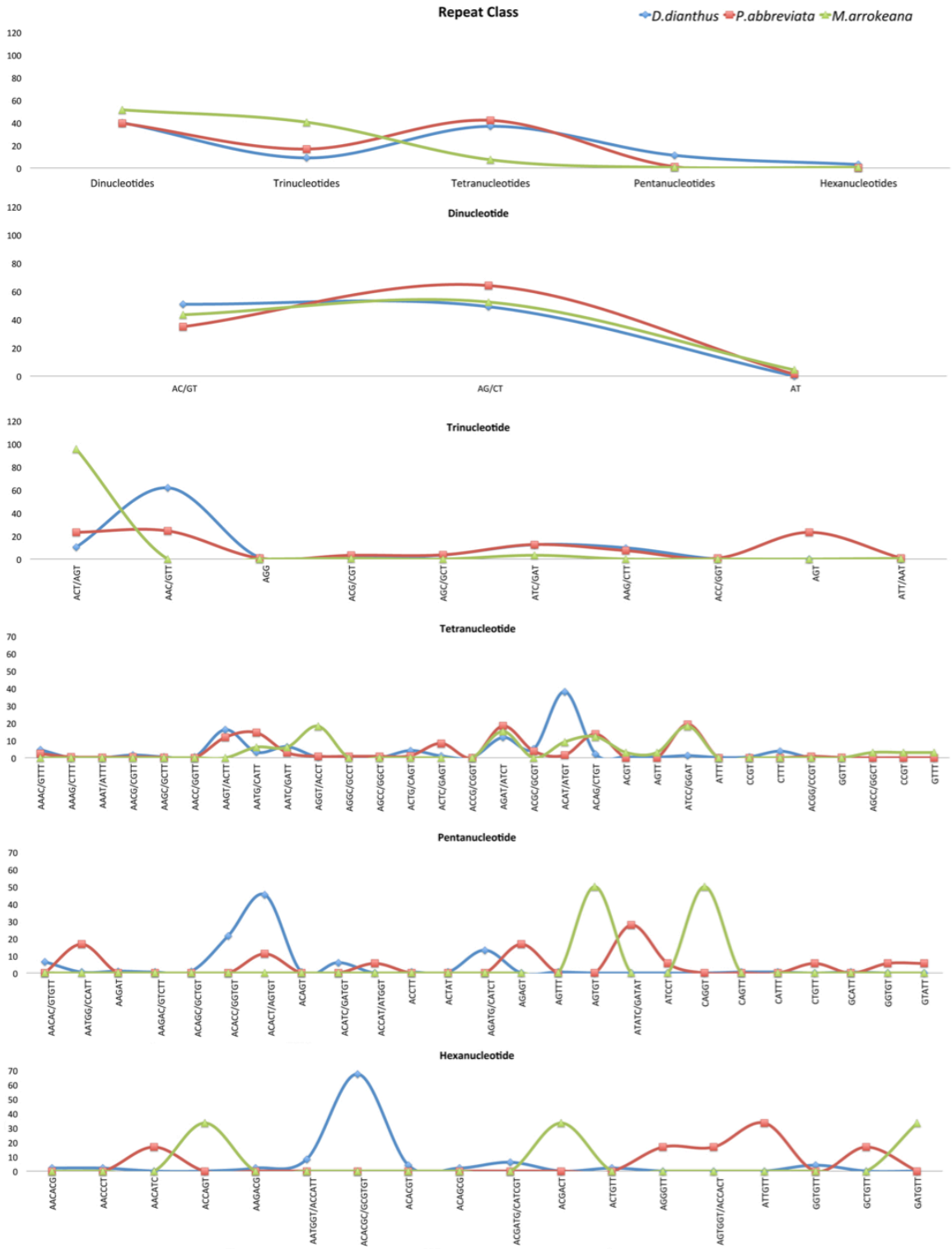

Figure 3.1. Frequency of repeat classes in three marine invertebrates (*Desmophyllum dianthus*, *Malacobdella arrokeana* and *Panopea abbreviata*).

Discussion

Genome-wide microsatellite characterisation, screening, and primer testing

Values of the number of alleles (N_A) per locus, observed (H_{Obs}) and expected (H_{Exp}) heterozygosities, and PIC were similar to that found in other corals (Casado-Amezúa *et al.* 2011; Le Goff and Rogers 2002; Molecular Ecology Resources Primer Development *et al.* 2010; Morrison *et al.* 2008; Underwood *et al.* 2006; Van Oppen *et al.* 2007).

As reviewed by Dakin and Avise (2004), null alleles are often reported in the population genetics literature, and several potential causes can lead to microsatellite null alleles: poor primer annealing due to nucleotide sequence divergence in one or both flanking primers (Kwok *et al.* 1990), differential amplification of size-variant alleles (Wattier *et al.* 1998), or PCR failure due to inconsistent or low DNA template quality (Gagneux *et al.* 1997; Garcia De Leon *et al.* 1998). Biological factors, such as the Wahlund effect of inbreeding or selection at or near a microsatellite locus, could also cause departures from HWE (Chakraborty *et al.* 1992). An alternative explanation could be due to the complex genetic structure of these organisms, due to large variances in reproductive success (and recruitment) and/or the possibility of asexual reproduction (Baus *et al.* 2005; Strathmann *et al.* 1984). For these reasons, Dakin and Avise (2004) concluded that, under realistic situations, although uncommon or rare (allele frequencies <0.2) microsatellite null alleles might cause a slight underestimation of the average exclusion probability at a given locus, they are unlikely to be of sufficient magnitude to warrant great concern. Even with an allele frequency threshold of <0.2, the presence of null alleles could not explain the departure from HWE for loci DdL58 and DdL84. Further analyses of more populations are necessary to better understand the potential causes of HWE departure for these loci. Neither significant scoring alleles nor evidence for large allele dropout were detected.

Nevertheless, an appreciable N_A and PIC are showed throughout the novel loci, which could be considered highly polymorphic and informative. Furthermore, the negative assortative mating revealed by the fixation index F_{IS} across the loci, confirmed that the most individuals are heterozygous and inbreeding process are not in act. Given these considerations, the novel loci developed here may be useful to infer population structure, larval dispersal, connectivity and gene flow across *D. dianthus* population.

Cross-species transferability

To date, the most common application of genetic studies in coral reef management is the use of population genetics data for implementing effective monitoring and management initiatives (e.g. design of Marine Protected Areas). Early discrepancies in coral population genetics data stem primarily from the difficulty in developing reliable, neutral genetic markers for studies on genetic connectivity in scleractinian corals (Lundgren 2011; Ridgway and Gates 2006).

In the last decade, 876 microsatellite markers were developed for only 2.5% of scleractinian species, representing 11 families and 18 genera, with an average of 12 microsatellites per species (Table 3.9) (source: National Center of Biotechnology Information NCBI- http://www.ncbi.nlm.nih.gov). The use of large scale, parallel-sequencing technologies may help overcome some of the technical limitations of coral genetic research by increasing the number of microsatellite markers, thus providing the conservation community with an extremely useful tool.

Among closely related taxa, microsatellites and their flanking sequences are often conserved, greatly facilitating their use as genetic markers (Schlötterer and Harr 2001). The fact that scorable genotyping loci were also working on coral species not phylogenetically closely related to *D. dianthus*, but informative for this species, is stressing the idea of the slow evolutionary rate that characterized corals, at least for the flanking regions. Nevertheless, the sizes of the different alleles genotyped were different for the different species tested, and thus, variability in the number of repetitions could be expected. Thus, the reliability of these markers can be considered for application to phylogeography or population genetic studies. The final proves will consist in sequencing all the obtained fragments for each locus, and amplifying them in different individuals (populations) to verify their information capacity.

Table 3.9. List of corals and numbers of related microsatellites available in GenBank (update May 2014).* Deep water coral.

Family	Genus	Species	No. Loci	Access Number	Reference
Acroporidae	*Acropora*	*cytherea*	48	GU136850-97	Concepcion et al. 2010
Acroporidae	*Acropora*	*digitifera*	6	AB451558, AB465609-13	Nakajima et al. 2009
Acroporidae	*Acropora*	*millepora*	11	EF014480-88; EF989161	van Oppen et al. 2007; Underwood et al. 2009
Acroporidae	*Acropora*	*muricata*	5	EU872430-34	Tang et al. 2010
Acroporidae	*Acropora*	*nobilis*	8	AB326925-32	Isomura et al. 2008
Acroporidae	*Acropora*	*palmata*	1	FJ460460	Baum et al. 2009
Acroporidae	*Acropora*	*sp.*	6	AB65614-19	Nakajima et al. 2009
Acroporidae	*Isopora*	*brueggemanni*	4	AB300380-83	Isomura et al. 2008
Acroporidae	*Montipora*	*capitata*	138	GU137008-144	Concepcion et al. 2010
Caryophylliidae	*Lophelia*	*pertusa**	36	AF455081-82, AY055734-42; EF577410-25; KF761605-12	Le Goff et al. 2002; Morrison et al. 2008; Morrison et al. (unpublished)
Dendrophyliidae	*Astroides*	*calycularis*	13	GQ292717-25, GQ496302-5	Casado-Amezua et al. 2010
Dendrophyliidae	*Eguchipsammia*	*fistula**	14	KC130924-37	Mughal et al. 2012
Euphyllidae	*Galaxea*	*fascicularis*	5	AB272099-103	Chen et al. 2013
Fungidae	*Fungia (Lobactis)*	*scutaria*	109	GU136898-7006	Concepcion et al. 2010
Incertae sedis	*Cladocora*	*caespitosa*	13	HM469895-907	Casado-Amezua et al. 2011
Merulinidae	*Goniastrea*	*favulus*	5	AY397652-56	Miller et al. 2004
Merulinidae	*Platygyra*	*acuta*	18	KC662261-78	Yang et al. 2014
Merulinidae	*Platygyra*	*daedalea*	5	AY397647-51	Miller et al. 2004
Montastraenidae	*Montastraea*	*annularis*	14	AF112346-51; AY395774-777	Lopez et al. 1999; Severance et al. 2004
Montastraenidae	*Montastraea*	*cavernosa*	5	AY445583-87, AY445590	Shearer et al. 2004
Montastraenidae	*Montastraea*	*franksi*	10	AF110121-29, AJ223626	Lopez et al. 1999
Montastraenidae	*Montastraea*	*faveolata*	7	AF110114-120	Lopez et al. 1999
Mussidae	*Favia*	*fragum*	15	EU181546-61	Carlon et al. 2008
Oculinidae	*Madrepora*	*oculata**	38	KF944310-47	Springmann et al. (unpublished)
Pocilloporidae	*Pocillopora*	*damicornis*	37	AB214372-81, AB329722, AB330134; DQ684672-77, EF120463-65	Hirose et al. ; Starger et al. 2007
Pocilloporidae	*Pocillopora*	*verrucosa*	5	AY397777-8, AY39780-82	Magalon et al. 2004
Pocilloporidae	*Seriatopora*	*hystrix*	17	AF320765-69; AY604005-6; DQ131572-81	Maier et al. 2001; Maier et al. 2005; Underwood et al. 2006
Pocilloporidae	*Stylophora*	*sp*	17	no available	Banguera-Hinestroza et al. 2013
Poritidae	*Porites*	*astreoides*	3	AY445585, AY445588-89	Shearer et al. 2004
Poritidae	*Porites*	*lobata*	142	GU137145-286	Concepcion et al. 2010
Poritidae	*Porites*	*lutea*	129	HQ435882-436001	Wang et al. (unpublished)
Poritidae	*Porites*	*rus*	7	HQ641440-6	Baranets et al. 2011

Repeat distribution across species

The pattern and characteristics of microsatellite distribution is species-specific and may reflect the divergence/evolutionary history of an organism, and their biological functions (Li *et al.* 2002; Li *et al.* 2004). The repeat distribution were examined in several eukaryotic taxonomic groups: primates, rodents, other mammals, no mammalian vertebrates, arthropods, nematodes, plants, yeast, and other fungi, but this study showed the first data for bivalves, nemertea and corals (Plough and Marko 2014; Tóth *et al.* 2000).

Next-generation sequencing is an extremely useful technology that can aid the development of large scale, suitable genetic markers for coral research. In this study, novel polymorphic microsatellite markers revealed high efficiency in individual identification and therefore, will be useful for a wide range of future studies investigating population structure, genetic diversity, and parental verification in the deep-sea coral *D. dianthus*. Cross-species amplifications provide potential markers for species in which none have yet been identified, and new or common ones with species of particular interest (e.g. *Lophelia pertusa)*.

Overall, given these advancements, we are now better prepared to gather more knowledge of the reproductive biology of *D. dianthus*, its dispersal abilities and the genetic structure and gene flow among different populations along its geographical distribution, which would be highly informative for any conservation and management plan.

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CHAPTER IV

Chapter adapted from:

Addamo AM, Miller K, Haüssermann V, Taviani M, Machordom A (In prep.) Global-scale genetic structuring and inferences on larval dispersal in *Desmophyllum dianthus* (Esper, 1794) (Cnidaria, Anthozoa, Scleractinia): two hemispheres in comparison.

Desmophyllum serpuliforme. Charles Joseph Gravier (1920). Madreporaires provenant des Campagnes des yachts Princesse-Alice et Hirondelle II (1893-1913).

Global-scale genetic structuring and inferences on larval dispersal in *Desmophyllum dianthus* (Esper, 1794) (Cnidaria, Anthozoa, Scleractinia): two hemispheres in comparison.

Abstract

Desmophyllum dianthus is one of the most common solitary corals among deep-sea species. Despite its wide distribution, it was not subjected to intensive biological studies, and information about reproduction strategy and larval dispersal is not yet available. In the present study, genetic structuring was analysed at broad spatial scale. Individuals of *D. dianthus* were collected from 13 localities distributed in the Mediterranean Sea and the Atlantic and Pacific oceans. The genetic variation was analysed using 30 microsatellites and significant levels of genetic differentiations were found between defined populations. Our results suggest that discrete larval dispersal (strongly depth-current-dependent) leads to peculiar phylogeographic structure, where populations of Chile and New Zealand showed own genetic characteristics, while gene flow is occurring between populations from Australia and Argentina.

Keywords: microsatellite, population structure, gene flow, larval dispersal, deep-sea coral, *Desmophyllum dianthus.*

Introduction

Cold-water coral reefs habitats play a major ecological role, forming locally enhanced biodiversity centres on the continental shelf and slope (Rogers 1999; Roberts *et al.* 2009). The deep-sea corals are extremely slow-growing organisms, living in some cases several centuries, providing not only an important habitat but also a valuable archive of past environmental conditions in their skeletal chemistry, an extraordinary information that would not be accessible otherwise (Risk *et al.* 2002; Roberts *et al.* 2009). Nevertheless, as many other marine ecosystems, deep-sea coral reefs have been affected by human activities, in particular fishery and the hydrocarbon industry which are

progressively pushing into deep waters (Risk *et al.* 2002; Roberts *et al.* 2009). Conservation strategies are closely related to the scientific knowledge about biology and ecology of target organisms, but unfortunately the biological understanding of corals inhabiting deep-seas is limited by logistical difficulties of studies at extreme depths, and indirect methods are used to infer about demography, biology and ecology of these organisms. Successfully results with deep-sea corals are reported in several studies (Le Goff-Vitry *et al.* 2004; Costantini *et al.* 2007; Costantini *et al.* 2011; Miller *et al.* 2011; Morrison *et al.* 2011; Dahl *et al.* 2012; Herrera *et al.* 2012). However, a large number of corals are still understudied and the widely distributed solitary species *Desmophyllum dianthus* (Esper, 1794) represents one of them. Even though no works on reproduction strategy and effective larval dispersal of *D. dianthus* have been published so far, a study on reproduction of Chilean specimens of *D. dianthus* has already started (R. Waller, pers. comm.). One exception is the study of Thresher *et al.* (2011), where inferences on recruitment periodicity, growth, and mortality rates were made by applying a modal analysis to the size frequency distribution of live-caught and sub-fossil specimens. Hypervariable molecular markers are demonstrated to provide a powerful tool to gain insight into molecular mechanisms of resilience and adaptation, and even if they are currently limited to individual or population scale studies, they are often applied to determine conservation and sustainable management strategy of coral reefs (Lundgren 2011). The core question of this study is providing information on population structure of *D. dianthus* based on 30 polymorphic microsatellites and 13 localities distributed in both northern and southern hemispheres. Potential larval dispersal, connectivity and their significance for conservations management are also discussed.

Material and Methods

Samples and study area

Coral tissue were sampled and preserved in absolute ethanol from specimens of *D. dianthus* collected during 18 cruises occurring between 2006 and 2012. Sampling took place in 13 localities: six in the Mediterranean Sea (315-1,350 m); three in the North Atlantic Ocean (500-1,069 m); one in the South Atlantic Ocean (757-1,629 m); and

three in the South Pacific Ocean (20-1,200 m) (Figure 4.1, Table 4.1). All necessary permits were obtained for the described field studies. This study did not involve endangered or protected species listed in the IUCN Red List of Threatened Species.

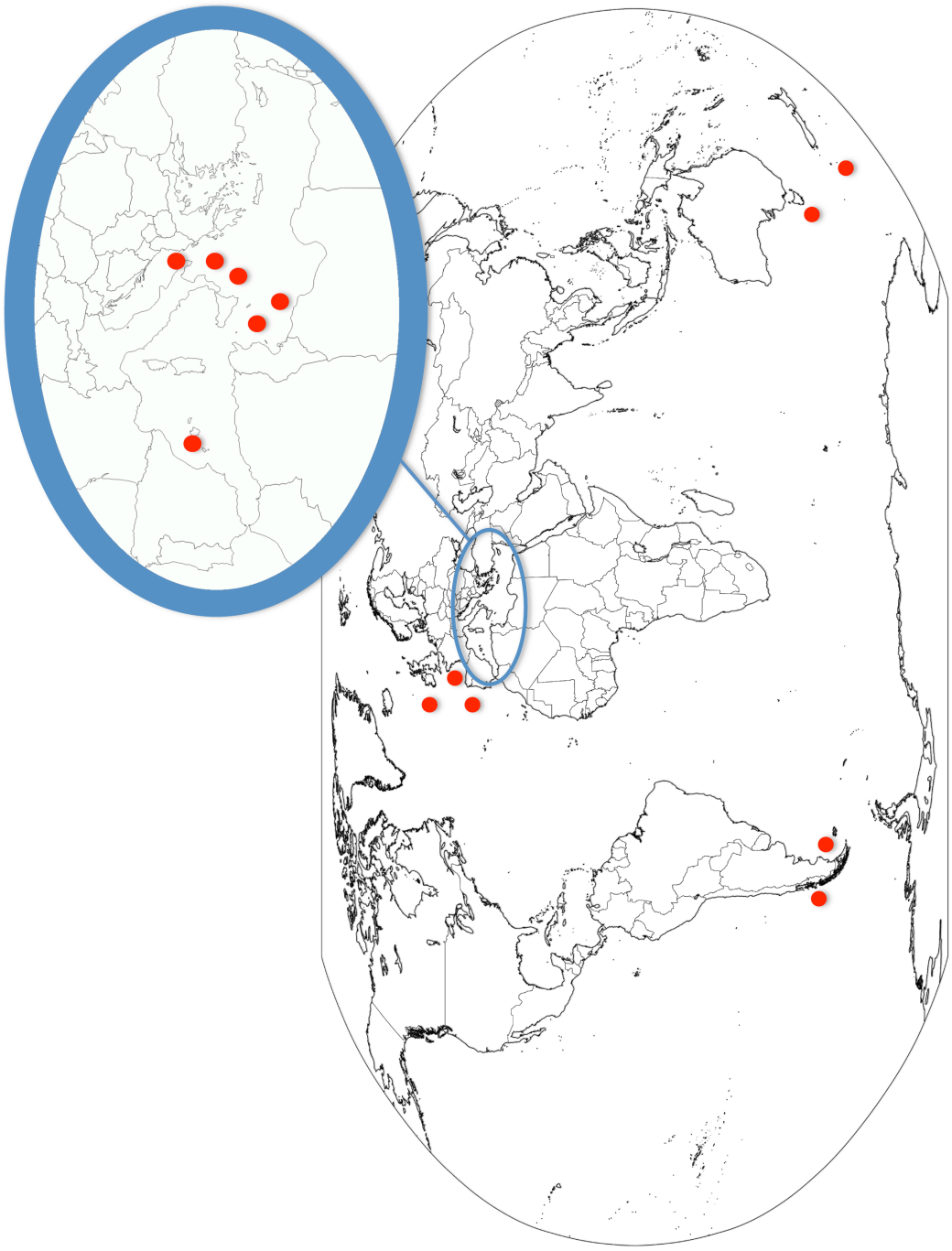

Figure 4.1. Map of *Desmophyllum dianthus* collection localities.

Table 4.1. Information on sampling technique, depth and geographic coordinates of sampling sites.

Code Locality	Expedition	Province/State	Precise Locality	Country	Range Depth	Lat (start)	Long (start)	Depth (start)	Lat (end)	Long (end)	Depth (end)	Technique	Period
Adriatic	MEMA12	South Adriatic Sea	off shore TRICASE (LECCE)	Italy	786	39°53.468'N	18°55.176'E					Grab	April-May 2012
Adriatic	M70	South Adriatic Sea	Bari Canyon	Italy	664-276	41°17'28.62"N	17°16'37.38"E	664	41°16'58.56"N	17°16'34.44"E	276	ROV Quest 4000	Summer 2006
Adriatic	M70	South Adriatic Sea	Bari Canyon	Italy	557-315	41°17'49.5"N	17°16'37.38"E	557	41°17'31.92"N	17°09'57.03"E	315	ROV Quest 4000	Summer 2006
Adriatic	M70	South Adriatic Sea	Dauno Seamount	Italy	unknown	41°33'1.47"N	17°28'5.91"E					ROV Quest 4000	Summer 2006
Adriatic	M70	South Adriatic Sea	Gondola Slide	Italy	710-674	41°43'30.48"N	17°02'47.64"E	710	41°43'10.62"N	17°03'39.3"E	674	ROV Quest 4000	Summer 2006
Adriatic	ALTRO	South Adriatic Sea	off Montenegro	Montenegro	480-470	41°38.885N	18°41.455E	480	41°38.885N	18°41.456E	470	ROV	April-May 2012
Adriatic	ALTRO	South Adriatic Sea	off Montenegro	Montenegro	480-490	41°38.885N	18°41.463E	480	41°38.906N	18°41.433E	490	ROV	April-May 2013
Ionian	M70	Ionian Sea	Gallipoli Escarpment	Italy	823--574	39°37'18.35'N'	18°04'47.51"E	823	39°37'30.66"N	18°05'1.08"E	574	ROV Quest 4000	Summer 2006
Ionian	CORSARO 26	Ionian Sea	Off Gallipoli	Italy	1097-1010	39°49'56.5"N	17°37'45.4"E	1097	39°50'38"N	17°37'30"E	1010	Rock dredge	April 2006
Ionian	CORSARO 73	Ionian Sea	SantaMaria di Leuca	Italy	671-679	39°37'29"N	18°39'05"E	671	39°38'07"N	18°40'23"E	679	Agassiz trawl	May 2006
Ionian	CORSARO 37	Ionian Sea	SantaMaria di Leuca	Italy	548-538	39°33'14"N	18°13'17"E	548	39°33'29"N	18°13'08"E	538	Epibenthic dredge	April 2006
Ionian	CORSARO 55	Ionian Sea	SantaMaria di Leuca	Italy	501-497	39°34'55.5"N	18°23'21.7"E	501	39°35'20.9"N	18°23'39"E	497	Epibenthic dredge	May 2006
Ionian	CORSARO 39	Ionian Sea	SantaMaria di Leuca	Italy	577-540	39°33'14.8"N	18°13'16.3"E	577	39°33'27.4"N	18°13'111"E	540	Epibenthic dredge	April 2006
Ionian-Calabria	Net	Ionian Sea	Roccella Ionica	Italy	unknow	38°18'2"N	16°29'8"E	-				Recupero rete	September 2006
Strait Sicily-Malta	MARCOS 43	Strait of Sicily	Malta	Malta	607-452	35°30'43.2"N	14°06'33.66"E	607	35°30'48.18"N	14°06'30.66"E	452	Epibenthic trawl	April 2007
Strait Sicily-Malta	MEDCOR	Strait of Sicily	Malta	Malta	690-462	35°30'28.66"N	14°11'2.74"E	690	35°31'0.98"N	14°10'26.034"E	462	Epibenthic dredge	December 2009
Strait Sicily	MEDCOR	Strait of Sicily	Gela	Italy	824-850	36°45'23.292"N	14°00'6.21"E	824	36°44'18.42"N	13°58'28.84"E	850	Epib.dredge Antolini	December 2009
Strait Sicily	MARCOS 36	Strait of Sicily	Linosa	Italy	819-403	35°46'0.6"N	13°02'36.54"E	819	35°45'46.74"N	13°02'19.02"E	403	Epibenthic trawl	April 2007
Strait Sicily	M70	Strait of Sicily	Urania Bank	Italy	654-440	36°50'19.56"N	13°09'20.03'E'	654	36°50'16.44"N	13°09'15"E	440	ROV Quest 4000	Summer 2006
Strait Sicily	MARCOS 22	Strait of Sicily	Urania Bank	Italy	626	36°50'20.46"N	13°09'21.72"E	626				Grab	April 2007
Catalan Slope	PROMETEO PR4M37	Catalan Slope	Cañon de Blanes - OpenSlope	Spain	1350	41°10'30"N	2°48'24"E	1350	41°10'30"N	2°48'24"E	1350	Marieta (Otter Trawl)	September 2007
Catalan Slope	HERMES 4 CORAL 8	Catalan Slope	Cap de Creus	Spain	unknown	42°19'09"N	3°19'19"E						September 2007
Atlantic-Galicia	ECOMARG-BANGAL V8	Galicia	Banco de Galicia	Spain	780	42°4501N	11°4658W	780					2009
Atlantic-Galicia	INDEMARES-BANGAL 07/11	Galicia	Banco de Galicia	Spain	903	42° 49.126' N	011° 46.592' W	903	42° 49.126' N	011° 46.592' W	903		August 2011
Atlantic-Galicia	INDEMARES-BANGAL 07/11	Galicia	Banco de Galicia	Spain	768	42° 44.209' N	011° 46.352' W	768	42° 44.209' N	011° 46.352' W	768		August 2011
Atlantic-Galicia	INDEMARES-BANGAL 07/10-07/11	Galicia	Banco de Galicia	Spain	750	42°13'19.2"N	11°23'40.2"W	750					
Atlantic-Cantabria	INDEMARES-AVILES 07/10	Cantabria	Cañon de Áviles	Spain	500	43.7291ºN	06.0901ªW	500					July-Aug 2010
Atlantic-Cantabria	INDEMARES-AVILES 04/10-05/11	Cantabria	Cañon de Áviles	Spain	500	43.7291ºN	06.0901ªW	500					
Atlantic-Ireland	EUROFLEETS-CWC Moira	Cork-Galway	Moira Mounts	Ireland	1069	51°26.33N	11°49.38W					Box Corer	June 2012
Atlantic-Ireland	EUROFLEETS-CWC Moira	Cork-Galway	Moira Mounts	Ireland	1058	51°26.58N	11°49.21W					Box Corer	June 2012
Atlantic-Ireland	EUROFLEETS-CWC Moira	Cork-Galway	Moira Mounts	Ireland	962	51°29.67N	11°49.14W					Box Corer	June 2012
Atlantic-Argentina	Patagonia 0108	off Argentina	Falkland Islands	Argentina	863	46°58'26.76"S	59°53'36.24"E					Trawl	October 2008
Atlantic-Argentina	Patagonia 0108	off Argentina	Falkland Islands	Argentina	817	46°56'9.6"S	60°0'36"E					Dredge	October 2008
Atlantic-Argentina	Patagonia 0108	off Argentina	Falkland Islands	Argentina	934	47°11'35.88"S	59°45'27.72"E					Trawl	November 2008
Atlantic-Argentina	Patagonia 0108	off Argentina	Falkland Islands	Argentina	757	47°16'58.8"S	59°57'39.6"E					Dredge	October 2008
Atlantic-Argentina	Patagonia 0209	off Argentina	Falkland Islands	Argentina	1581	44°10'4.8"S	59°10'58.8"E					Dredge	April 2009
Atlantic-Argentina	Patagonia 0209	off Argentina	Falkland Islands	Argentina	1478	44°19'8.76"S	59°22'22.08"E					Trawl	February 2009
Atlantic-Argentina	Patagonia 0209	off Argentina	Falkland Islands	Argentina	1244	43°17'5.64"S	59°0'3.24"E					Trawl	February 2009
Atlantic-Argentina	Patagonia 0209	off Argentina	Falkland Islands	Argentina	1500	43°58'51.6"S	59°17'27.6"E					Dredge	April 2009
Atlantic-Argentina	Patagonia 0209	off Argentina	Falkland Islands	Argentina	1529	43°0'21.6"S	58°44'38.4"E					Dredge	May 2009
Atlantic-Argentina	Patagonia 0209	off Argentina	Falkland Islands	Argentina	1629	44°8'24"S	59°22'58.8E					Dredge	March 2009
Atlantic-Argentina	ANTXIX/5STN153	off Argentina	Burwood Bank	Argentina	300	54° 0'30"S	60° 0'30'W	300					
Chile-Comau	AMACA	Pitipalena fijord- region XI	Isla Jaime	Chile	23	43°46.27S	72°55.045W					SCUBA	February 2012
Chile-Comau	AMACA-HuinayAquaria	Comau fijord-region X	Isla Lilihuapi	Chile		42°22'10"S	72°27'18"W					SCUBA	February 2012
Chile-Comau	AMACA-Huinay	Comau fijord-region X	Isla Lilihuapi	Chile		42°22'10"S	72°27'18"W					SCUBA	February 2012
Chile-Comau		Comau fijord-region X	Isla Lilihuapi	Chile		42°22'10"S	72°27'18"W					SCUBA	August 2012
Australia	Southern Surveyor SS02/2007	Tasmania	DoryHIll W	Australia	1100-1200	44°19'34.54"S	147°7'8.15"W	1100	44°19'33.82"S	147°6'50.44"W	1200	Gear Sled	April 2007
New Zealand	TAN0803	Macquaire Ridge		New Zealand	385	51°3'39.6"S	161°58'40.8"W						April 2008

Microsatellite genotyping and characterisation

Total genomic DNA was extracted from the mesenteric tissue of 358 *D. dianthus* specimens using the QIAGEN BioSprint 15 DNA Blood Kit (Qiagen Iberia S. L., Madrid), with slight modifications, including the optional RNase treatment and an extended period of proteinase K lysis (overnight incubation at 55 °C). DNA concentration was quantified using the Qubit 2.0 Fluorometer and diluted to a final concentration of 2 ng/μl. Thirty-one microsatellite loci developed for *D. dianthus* (Chapter III; K. Miller pers. comm.) were organized in 1 tetraplex, 7 triplex, and 3 diplex reactions by Multiplex Manager 1.0 (Holloley and Geerts 2009) and analysed in each sample. Multiplex PCRs were performed using 1X Qiagen Multiplex PCR Master Mix (Qiagen, Hilden, Germany), following the PCR conditions described in Chapter III. Fluorescently labelled PCR products were run on an ABI PRISM 3730 DNA Sequencer (Applied Biosystems), scored using the GeneScan-500 (LIZ) size standard, and analysed with GeneMapper software (Applied Biosystems). Estimates of null allele frequency, error scoring, and large allele dropout were calculated with Brookfield-1 method (Brookfield 1996) using Micro-Checker (Van Oosterhout *et al.* 2004). Due to possible asexual reproduction of corals (e.g. via budding) and their close relationship in space that induces putative multiple sampling of the "same" individual, identification of individuals with identical multilocus genotype, through the index of probability of identity (i.e. PI, the probability of two individuals sharing the same genotype) was calculated using GenAlEx 6.5 (Peakall and Smouse 2012).

Genetic diversity

Genetic variability within samples was estimated as observed (H_{Obs}) and expected (H_{Exp}) heterozygosity. Significant departure from Hardy-Weinberg Equilibrium (HWE) per each locus was also calculated. The inbreeding coefficients of individual relative to each subpopulation (F_{IS}) and to the total population (F_{IT}), and the effect of subpopulation compared to the total population (F_{ST}) were estimated for each locus separately and for all loci. Moreover, allelic richness (N_A) and private allelic richness (P_A) were calculated for each locality. Computations were made using GenAlEx 6.5. Genotypic linkages disequilibrium (LD) per each pair of locus at each sampled locality was computed under exact test using Genepop 4.1 (Raymond and Rousset 1995;

Rousset 2008) and analysis of significance was tested with Markov Chain Monte Carlo (MCMC). Sequential Holm-Bonferroni correction (Holm 1979) was applied to the multiple tests. Comparison detection of genetic markers exhibiting locus-specific (outliers) effects associated to non-neutral selection was made with coalescent, Bayesian and hierarchical approaches using LOSITAN (Antao *et al.* 2008), BayeScan v2.01 (Fischer *et al.* 2011) and Arlequin v.3.5 (Excoffier and Lischer 2010) respectively.

Genetic structure

To investigate population structure, number of genetic clusters (K) from multilocus genotype data was inferred with a Bayesian model-based approach implemented in Structure v2.3.4 (Falush *et al.* 2003). Setting for all runs included 100,000 MCMC interactions after a burn-in of 10,000 iterations. Ten independent chains were run to test each value of K from 1 to 20. To detect the best-fit number of genetic cluster representing the genetic discontinuity of the data, results from S Structure were processed in S Structure Harvester (Earl and vonHoldt 2012), and the highest mean lnPr(X|K) (Pritchard *et al.* 2000) and ΔK (Evanno *et al.* 2005) were considered to evaluate the optimum value of K. Each cluster identified in the initial Structure run was analysed separately using the same settings to identify potential within-cluster structure (Evanno *et al.* 2005). Pairwise genetic distances (F_{ST}) between clusters suggested by Structure and population assignment were calculated taking into account differences in allele size using GenAlEx 6.5. Microsatellite data were also subjected to hierarchical analyses of molecular variance (AMOVA), implemented in Arlequin 3.5, including two hierarchical levels: among and within populations.

Genetic-spatial correlation and demographic parameters

Isolation by distance was examined testing the correlation between genetic and geographic distances between localities pairs. The regression of the linearised F_{ST} (F_{ST}/ (1-F_{ST})) versus marine geographic distance (Km) was tested using Mantel test implemented in GenAlEX 6.5. Marine geographic distances between localities were calculated using Google Earth (Google Inc. 2009) and considering the straightest marine route. To define a phylogeographic structure, Spatial Analysis of Molecular Variance was performed using SAMOVA 1.0. This method maximizes the proportion of genetic variance due to differences between a user-defined number of groups (K),

and assigns localities to groups, considering that they must be geographically adjacent and genetically homogeneous (Dupanloup *et al.* 2002). Phylogeographic reconstruction, based on Nei pairwise genetic distances among the 13 localities were performed under Neighbour-Joining criteria implemented in Phylip v3.65 (Felsenstein 2005).

Results

A total of 358 specimens were analysed using 31 microsatellite loci. All loci were successfully genotyped in all populations, but one was excluded due to PCR failure in more than 30% of total individuals (DdL97). The mean probability of two randomly sampled corals having identical genotypes, based on the first 4 loci (DdL7, DdL13, DdL16 and DdL22), was estimated at 7.7×10^{-7} with a mean cumulative probability of exclusion (PE) of 99.61%, 97.59%, and 99.99% when, respectively, the genotypes of both parents were known, when only one parent was known and when two putative parents were excluded. In the extreme situation that all individuals were in full-sibling relationships (considering the combination of all 30 loci), the probability of identity (PI) was estimated at 7.7×10^{-7}. Therefore, this four microsatellites panel is theoretically sufficient for individual identification of any coral in the analysed populations. Shared genotypes were considered as belonging to the same individual and only one matching multilocus genotypes was found, thus following analyses were performed with a total of 357 different multilocus genotypes.

Neither significant scoring alleles errors nor evidence for large allele dropouts were detected. Null allele frequencies calculated using the Brookfield-1 method determined false homozygotes genotypes for 10 loci (B118, C102, DdL7, DdL22, DdL41, DdL51, DdL58, DdL84, DdL90, and DdL109). Others potential causes (e.g. strong inbreeding or selection for or against a certain allele, Wahlund effect, or linkage disequilibrium) are likely for 4 (B118, DdL7, DdL58, DdL84) of those 10 loci where the presence of null alleles (e.g. caused by amplification failure, or large alleles dropout) could not explain the excess of homozygosis and consequent departure from HWE (Tables 4.2, 4.3 and 4.4) proportions. Therefore, Brookfield-1 adjustments were applied to loci, whose null alleles were detected, and outlier and linkage disequilibrium tests were performed in order to evaluate the selective neutrality and gene association of each locus, respectively,

and determine reasons, which could explain the homozygote excess detected for loci B118, DdL7, DdL58, DdL84. Outlier Tests computed under Bayesian, coalescent and hierarchical criteria determined inconsistent results: none of the analyses were simultaneously significant for all loci, since any locus was putatively identified as gene under selective pressure, hence the hypothesis that loci could be under selection have been rejected. Tests for linkage disequilibrium (LD) yielded 1.30% significant tests of linked loci (after sequential Holm-Bonferroni correction) among a total of 5,655 pairwise comparisons. Due to a lack of homogeneity of significant analyses for all loci in all populations, physical linkage detected in 74 pairwise comparisons has been rejected. Thus, null alleles frequencies were considered in the data set for successive analyses.

All loci were polymorphic with a total number of alleles ranging from 3, for DdL86, to 21, for C6, with a mean value of 9 alleles per locus. Ratio of allelic richness to private allelic richness per each sampled locality showed a heterogeneity pattern, in which localities from the southern hemisphere stood out for high private alleles frequency (Figure 4.2). The observed heterozygosity (H_{Obs}) for the total sample varied between 0.29 and 0.89 (for DdL58 and C6 respectively), with a mean value over loci of 0.59 ± 0.011; and expected heterozygosity (H_{Exp}) varied between 0.41 and 0.92 (for DdL86 and C6 respectively), with a mean value over loci of 0.72 ± 0.009 (Table 4.2). Locus values of the F_{IS} varied between -0.041 and 0.588 (for DdL82 and DdL58 respectively), with a mean value over loci of 0.181 ± 0.035, indicating a global deficit of heterozygotes (Table 4.2). By population, the H_{Obs} showed the smallest value in the Chilean population (0.52 ± 0.05) and the greater one in the Adriatic population (0.63 ± 0.04), while uH_{Exp} varied between 0.63 ± 0.05 (Chile-Comau) and 0.82 ± 0.02 (Atlantic-Cantabrian) (Table 4.3)

Table 4.2. Summary statistics for each locus. Heterozygosity, Fstatistics and polymorphism by population for codominant data.N=sample size; N_A= number of alleles; N_E= number of effective allelee; I= Shannon's Information Index; H_{Obs}= observed heterozygosity; H_{Exp}= expected heterozygosity uH_{Exp}= unbiased expected heterozygosity; F= fixation index.

	N		N_A		N_E		I		H_{Obs}		H_{Exp}		uH_{Exp}		F	
Locus	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**
B4	25,615	3,033	13,385	0,636	6,270	0,694	2,108	0,077	0,749	0,039	0,815	0,021	0,837	0,023	0,078	0,045
B9	25,846	3,138	11,000	0,801	5,097	0,426	1,911	0,071	0,784	0,021	0,786	0,019	0,807	0,020	-0,003	0,033
B118	25,846	3,119	17,462	1,113	10,837	0,572	2,573	0,064	0,648	0,025	0,904	0,006	0,928	0,004	0,284	0,027
C6	26,538	3,218	21,231	2,013	13,390	1,235	2,759	0,089	0,894	0,026	0,919	0,006	0,942	0,005	0,026	0,030
C102	25,077	3,016	9,538	0,945	4,252	0,481	1,690	0,117	0,476	0,039	0,727	0,030	0,746	0,030	0,355	0,041
C107	26,308	3,193	3,846	0,504	2,149	0,253	0,849	0,122	0,413	0,055	0,465	0,052	0,478	0,055	0,113	0,049
L7	23,769	2,987	14,615	1,318	8,080	0,796	2,301	0,095	0,457	0,040	0,857	0,019	0,881	0,020	0,469	0,043
L13	26,308	3,241	9,692	0,559	4,330	0,385	1,737	0,058	0,732	0,032	0,751	0,018	0,771	0,020	0,028	0,030
L16	26,385	3,204	9,462	1,078	4,122	0,543	1,569	0,193	0,662	0,078	0,648	0,077	0,665	0,079	-0,029	0,019
L22	24,769	3,237	6,923	0,780	4,277	0,639	1,405	0,203	0,394	0,055	0,619	0,087	0,638	0,090	0,278	0,066
L24	26,000	3,158	11,923	0,763	6,863	0,512	2,136	0,060	0,592	0,022	0,846	0,009	0,868	0,009	0,300	0,026
L34B	26,154	3,236	15,769	1,557	7,576	0,966	2,231	0,125	0,777	0,044	0,831	0,033	0,853	0,034	0,068	0,034
L41	25,846	3,204	10,538	1,202	6,378	0,769	1,909	0,179	0,641	0,075	0,774	0,064	0,795	0,066	0,155	0,068
L50	26,000	3,226	17,385	1,385	8,728	0,987	2,437	0,089	0,876	0,022	0,866	0,017	0,889	0,018	-0,010	0,012
L56	26,077	3,187	7,154	0,373	3,615	0,204	1,499	0,047	0,720	0,029	0,714	0,015	0,732	0,015	-0,009	0,034
L57	23,000	2,785	6,462	0,550	4,007	0,225	1,548	0,053	0,312	0,033	0,741	0,015	0,762	0,015	0,583	0,041
L58	20,077	2,510	7,769	0,611	4,189	0,497	1,583	0,121	0,290	0,044	0,704	0,046	0,729	0,048	0,556	0,079
L73	25,231	3,032	6,615	0,401	3,421	0,209	1,414	0,053	0,659	0,035	0,693	0,020	0,712	0,019	0,053	0,038
L82	26,000	3,172	7,923	0,329	4,483	0,325	1,644	0,082	0,785	0,031	0,754	0,028	0,775	0,030	-0,048	0,032
L83	25,923	3,187	8,154	0,576	4,109	0,391	1,607	0,080	0,733	0,035	0,734	0,021	0,753	0,021	-0,001	0,046
L84	24,077	2,863	11,000	1,050	5,837	0,654	1,935	0,123	0,431	0,029	0,790	0,031	0,811	0,031	0,446	0,043
L86	25,692	3,187	3,538	0,243	1,806	0,131	0,737	0,067	0,367	0,040	0,413	0,041	0,424	0,043	0,080	0,067
L90	25,462	3,081	7,462	0,685	3,126	0,304	1,369	0,120	0,357	0,041	0,623	0,052	0,641	0,054	0,425	0,046
L91	25,923	3,171	6,385	0,513	3,604	0,246	1,468	0,066	0,549	0,032	0,708	0,018	0,726	0,018	0,224	0,042
L96	25,385	3,169	7,769	0,469	3,343	0,268	1,468	0,063	0,649	0,025	0,679	0,024	0,697	0,025	0,039	0,034
L98	26,000	3,144	4,462	0,369	2,029	0,153	0,902	0,095	0,419	0,043	0,465	0,049	0,477	0,050	0,067	0,053
L100	25,615	3,143	6,077	0,473	2,907	0,180	1,275	0,069	0,634	0,042	0,634	0,032	0,650	0,033	0,000	0,048
L102	26,231	3,177	8,846	0,697	4,541	0,357	1,713	0,081	0,742	0,037	0,760	0,022	0,779	0,021	0,023	0,041
L107	23,385	3,245	10,000	0,899	6,470	0,515	1,986	0,083	0,576	0,059	0,833	0,013	0,858	0,012	0,313	0,070
L109	24,769	2,903	6,846	0,576	3,616	0,328	1,470	0,084	0,454	0,048	0,703	0,020	0,723	0,021	0,351	0,069
Total	25,310	0,550	9,641	0,262	5,115	0,161	1,708	0,030	0,592	0,011	0,725	0,009	0,745	0,009	0,174	0,013

Table 4.3. F-Statistics and estimates of measure of gene flow (Nm) over all sampling sites for each locus. F_{IS}= inbreeding coefficient of an individual with respect to the local subpopulation; F_{IT}= the inbreeding coefficient of an individual relative to the total population; F_{ST}= the average inbreeding coefficient of subpopulations relative to the total population.

Locus	F_{IS}	F_{IT}	F_{ST}	Nm
B4	0,082	0,144	0,068	3,435
B9	0,002	0,088	0,086	2,657
B118	0,283	0,313	0,041	5,782
C6	0,027	0,062	0,036	6,741
C102	0,346	0,384	0,059	4,017
C107	0,112	0,318	0,232	0,828
L7	0,467	0,513	0,086	2,661
L13	0,026	0,109	0,085	2,676
L16	-0,021	0,160	0,178	1,156
L22	0,364	0,522	0,249	0,755
L24	0,300	0,325	0,036	6,652
L34B	0,066	0,167	0,108	2,056
L41	0,172	0,288	0,140	1,541
L50	-0,011	0,028	0,038	6,276
L56	-0,009	0,035	0,044	5,468
L57	0,578	0,603	0,059	3,977
L58	0,588	0,655	0,162	1,296
L73	0,050	0,092	0,043	5,508
L82	-0,041	0,086	0,122	1,802
L83	0,001	0,102	0,102	2,210
L84	0,454	0,520	0,121	1,815
L86	0,111	0,454	0,386	0,398
L90	0,426	0,540	0,198	1,015
L91	0,225	0,293	0,088	2,596
L96	0,044	0,126	0,085	2,691
L98	0,099	0,349	0,277	0,652
L100	-0,001	0,154	0,155	1,364
L102	0,023	0,129	0,109	2,052
L107	0,308	0,361	0,077	2,997
L109	0,355	0,449	0,145	1,469
Mean	0,181	0,279	0,120	2,818
SE	0,035	0,034	0,015	0,347

Table 4.4. Summary statistics for each sampled site of *Desmophyllum dianthus*. N=sample size; N_A= number of alleles; N_E= number of effective allelee; I= Shannon's Information Index; H_{Obs}= observed heterozygosity; H_{Exp}= expected heterozygosity; uH_{Exp}= unbiased expected heterozygosity; F= fixation index.

Sampling Site	Value	N	N_A	N_E	I	H_{Obs}	H_{Exp}	uH_{Exp}	F
Adriatic	**Mean**	23,900	9,433	5,230	1,762	0,631	0,749	0,765	0,146
	SE	0,427	0,745	0,523	0,096	0,036	0,025	0,026	0,045
Ionian	**Mean**	39,267	11,333	5,321	1,809	0,607	0,749	0,759	0,177
	SE	0,593	1,003	0,549	0,102	0,035	0,027	0,027	0,044
Ionian-Calabria	**Mean**	30,133	10,367	5,090	1,776	0,621	0,747	0,759	0,157
	SE	0,348	0,914	0,476	0,097	0,034	0,026	0,027	0,039
Strait Sicily-Malta	**Mean**	30,733	10,433	4,962	1,753	0,615	0,741	0,753	0,157
	SE	0,335	0,951	0,510	0,095	0,036	0,025	0,025	0,046
Strait Sicily	**Mean**	42,067	11,733	5,553	1,862	0,587	0,765	0,774	0,239
	SE	0,489	0,995	0,603	0,097	0,039	0,023	0,023	0,044
Catalan Slope	**Mean**	12,667	7,500	4,776	1,624	0,621	0,720	0,751	0,130
	SE	0,241	0,575	0,479	0,096	0,042	0,032	0,033	0,046
Atlantic-Galicia	**Mean**	20,300	9,267	5,038	1,764	0,582	0,761	0,780	0,234
	SE	0,296	0,761	0,452	0,083	0,033	0,019	0,020	0,040
Atlantic-Cantabria	**Mean**	14,367	9,800	5,934	1,894	0,604	0,792	0,821	0,246
	SE	0,182	0,731	0,554	0,085	0,034	0,016	0,017	0,038
Atlantic-Ireland	**Mean**	6,933	5,867	4,344	1,509	0,599	0,717	0,773	0,153
	SE	0,046	0,395	0,413	0,076	0,041	0,021	0,023	0,057
Atlantic-Argentina	**Mean**	12,533	7,567	4,471	1,530	0,566	0,672	0,700	0,157
	SE	0,171	0,667	0,478	0,117	0,053	0,040	0,042	0,055
Chile-Comau	**Mean**	34,000	9,967	4,800	1,491	0,514	0,621	0,630	0,146
	SE	0,557	1,340	0,890	0,150	0,051	0,051	0,052	0,044
Australia	**Mean**	30,933	10,533	4,975	1,609	0,571	0,666	0,677	0,128
	SE	0,346	1,087	0,661	0,136	0,043	0,041	0,042	0,038
New Zealand	**Mean**	31,200	11,533	6,002	1,818	0,584	0,728	0,740	0,190
	SE	0,435	1,173	0,788	0,130	0,046	0,039	0,040	0,048
Total	**Mean**	25,310	9,641	5,115	1,708	0,592	0,725	0,745	0,174
	SE	0,550	0,262	0,161	0,030	0,011	0,009	0,009	0,013

Analyses in Structure suggested the microsatellite data were best explained by two genetic clusters of *D. dianthus* localities (K= 2), corresponding to geographic area as follows: northern [A] and southern hemisphere [B]. Additional hierarchical Structure runs were separately performed for each of these two groups that represent the localities belonging to Mediterranean Sea-North Atlantic Ocean [A], and the localities in South Atlantic Ocean-Pacific Ocean [B]. Sub-structuring was detected in both hemispheres: northern hemisphere was further subdivided in two genetic clusters (K= 2): Mediterranean Sea-Galicia [A1] and Ireland-Cantabria [A2], while the southern hemisphere was further subdivided in three genetic clusters (K= 3): Argentina-Australia [B1], New Zealand [B2], and Chile [B3] (Table 4.5, Figure 4.3).

Table 4.5. Average proportion of membership by locality for the clusters identified by Structure (Falush *et al.* 2003). K= genetic cluster; No= number assigned to the population. For [A], [B], [A1], [A4], [B1], [B2], [B3] see the main text.

	Cluster K=2			**Subcluster K=2**			**Subcluster K=3**			
Sampling Site	**No**	[A]	[B]	**No**	[A1]	[A2]	**No**	[B1]	[B2]	[B3]
Adriatic	1	0,989	0,011	1	0,983	0,017				
Ionian	2	0,97	0,03	2	0,965	0,035				
Ionian-Calabria	3	0,985	0,015	3	0,983	0,017				
Strait Sicily-Malta	4	0,99	0,01	4	0,989	0,011				
Strait Sicily	5	0,917	0,083	5	0,901	0,099				
Catalan Slope	6	0,996	0,004	6	0,993	0,007				
Atlantic-Galicia	7	0,897	0,103	7	0,871	0,129				
Atlantic-Cantabria	8	0,509	0,491	8	0,446	0,554				
Atlantic-Ireland	9	0,657	0,343	9	0,482	0,518				
Atlantic-Argentina	10	0,016	0,984				1	0,065	0,013	0,922
Chile-Comau	11	0,006	0,994				2	0,015	0,978	0,007
Australia	12	0,013	0,987				3	0,006	0,01	0,984
New Zealand	13	0,039	0,961				4	0,932	0,032	0,036

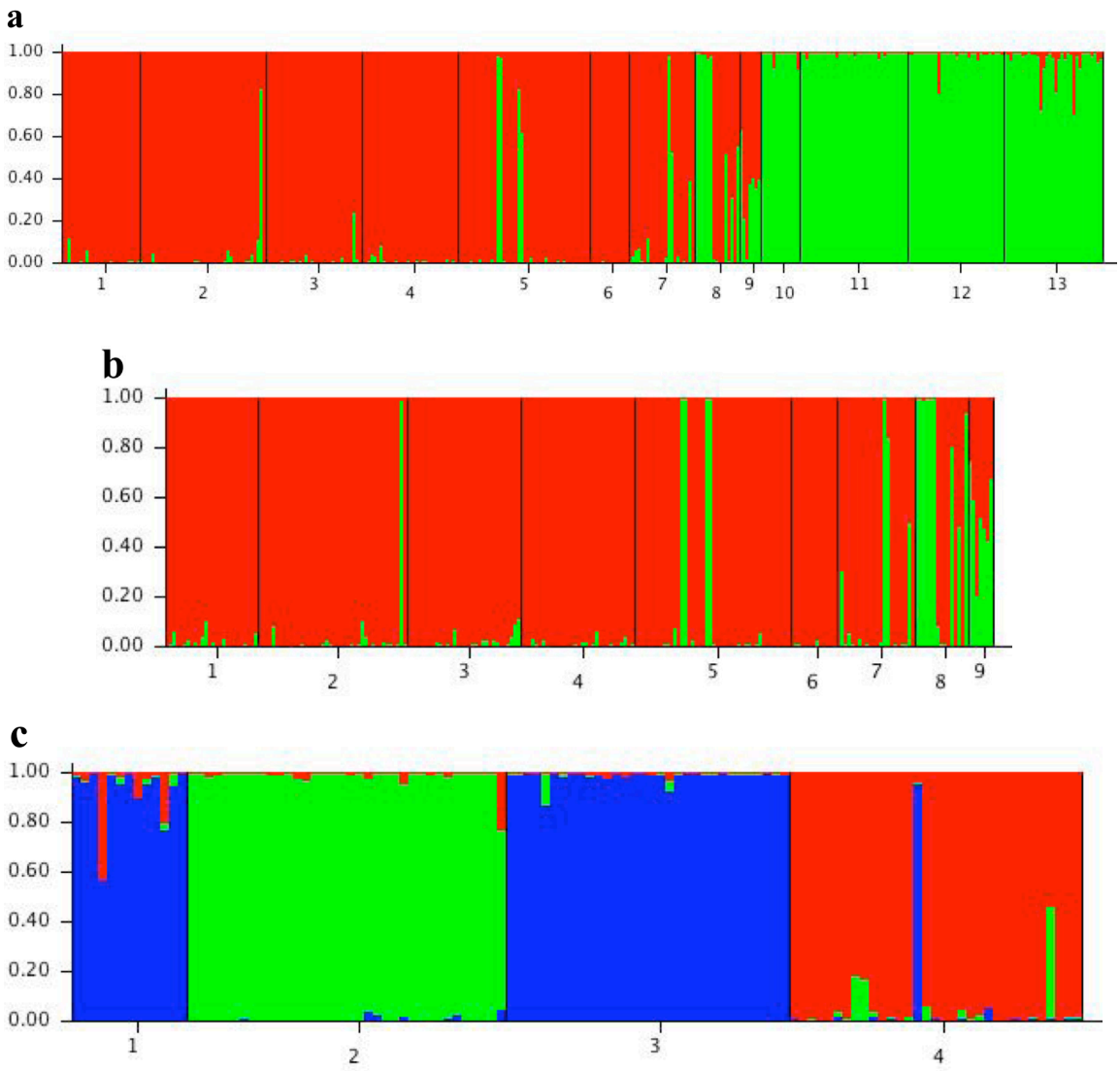

Figure 4.3. Proportional membership of *Desmophyllum dianthus* individuals from sequential cluster analyses using Structure. The clusters are shown with the vertical bars representing each individual broken into colored segments based on the proportion of the genome estimated to have originated from each cluster. Localities (**a**) were structured initially in two clusters (K= 2): northern hemisphere (red) and southern hemisphere (green). Sampling sites from northern hemisphere (**b**) no presented structure (K= 1), whereas the southern hemisphere cluster (**c**) contained additional structuring (K= 3) identified as Chile cluster (green), Argentina-Australia (blue), and New Zealand (red).

Quantitative estimates of hierarchical gene diversity (AMOVA) indicated an appreciable genetic population structure with a total differentiation index F_{ST} of 0.146 (p-value= 0.000), whereof 85.41% of variation was observed within localities, much more than the percentage variation observed among populations (14.59%) (Table 4.6). Test for estimating specimen assignment were performed using GenAlEx 6.5 obtaining results in concordance with population defined by Structure: only 17.6% of individuals

from Mediterranean and North Atlantic was assigned to own populations, and 82.4% were assigned to others from same marine area. Individuals from Chile and Australia were assigned to self-population with 100%, whiles New Zealand and Argentina showed respectively 93% and 84% of assignment to self-population (Table 4.7).

Table 4.6. Results from analysis of molecular variance (AMOVA) among clusters suggested by Structure.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among populations	4	543,976	1,25743 Va	14,59
Within populations	689	5073,151	7,36306 Vb	85,41
Total	693	5617,127	8,62049	

Fixation Index FST: 0,14586

Table 4.7. Summary of Individual Assignment Outcomes to 'Self' or 'Other' Sampling Site (With Leave One Out Option).

Sampling Site	Self Site	Other Site
Adriatic	8	18
Ionian	4	38
Ionian-Calabria	11	21
Strait Sicily-Malta	6	26
Strait Sicily	9	35
Catalan Slope		13
Atlantic-Galicia	2	20
Atlantic-Cantabria		15
Atlantic-Ireland	1	6
Atlantic-Argentina	11	2
Chile-Comau	36	
Australia	32	
New Zealand	31	2
Total	151	196
Percentage	43,52	56,48

To investigate phylogeographic structure, localities were tested for several clusters of populations (from 1 to 7) using SAMOVA 1.0 and in concordance with results produced by Structure, the initial 13 localities were better defined as 5 groups of populations geographically homogeneous and maximally differentiated from each other, showing clear genetic barrier between each other (F_{ST}= 0.16, F_{SC}= 0.01; F_{CT}= 0.15, p-values highly significant). F_{CT} is the index that will be associated with possible barriers (Dupanloup *et al.* 2002). Additional simulated annealing SAMOVA runs were performed including only Mediterranean and North Atlantic Ocean, and three phylogeographic groups were detected: Ireland, Cantabria, and Mediterranean Sea-

Galicia (F_{ST}= 0.05, F_{SC}= 0.002, p-values highly significant), separated each other by semipermeable genetic barriers (F_{CT}= 0.05, p-value= 0.03). Gene flow across semipermeable genetic barriers was also shown between Catalan Slope (NW Mediterranean Sea) and Central Mediterranean localities (Figure 4.4).

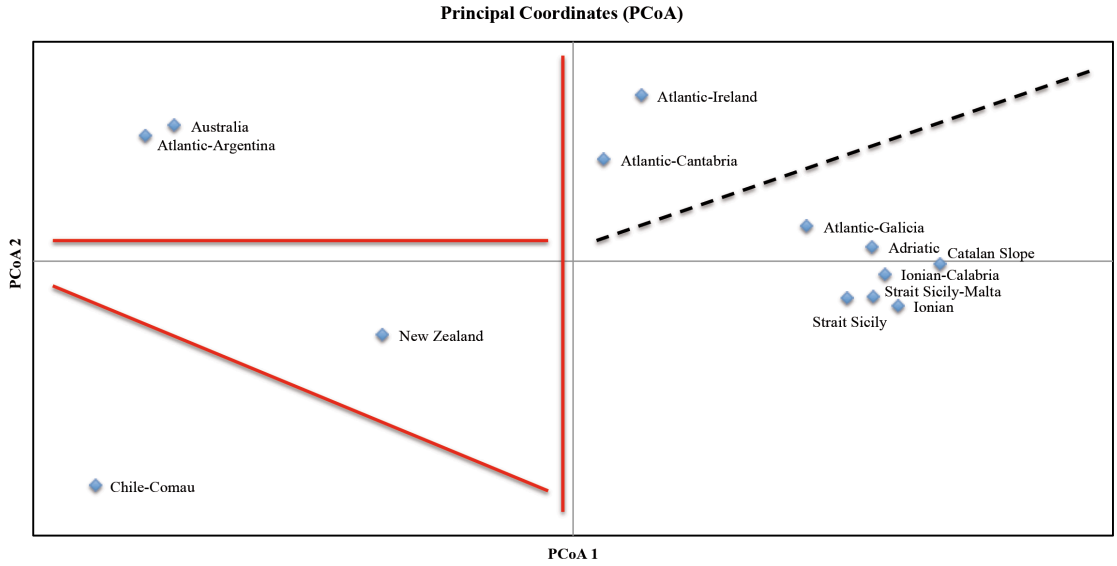

Figure 4.4. Plot of principal coordinates analyses of all microsatellite data, classified by sampling sites, with corresponding impermeable (red line) and semipermeable (black dotted line) genetic barriers estimated using SAMOVA.

Moreover, the phylogeographic topology, where populations are clustered by genetic similarity (Figure 4.5), showed a first dichotomy between specimens belonging to northern and southern areas. In the first group, there was a cluster that included the Mediterranean localities, and Galicia was its more closely related sample site; Ireland and Cantabria were consecutively clustered to the cited localities. In the southern group, Argentina and Australia samples were clustered together, and after the Chilean locality grouped with a long branch to them. Finally, New Zealand appeared in the base of the tree, but forced to its outgroup condition.

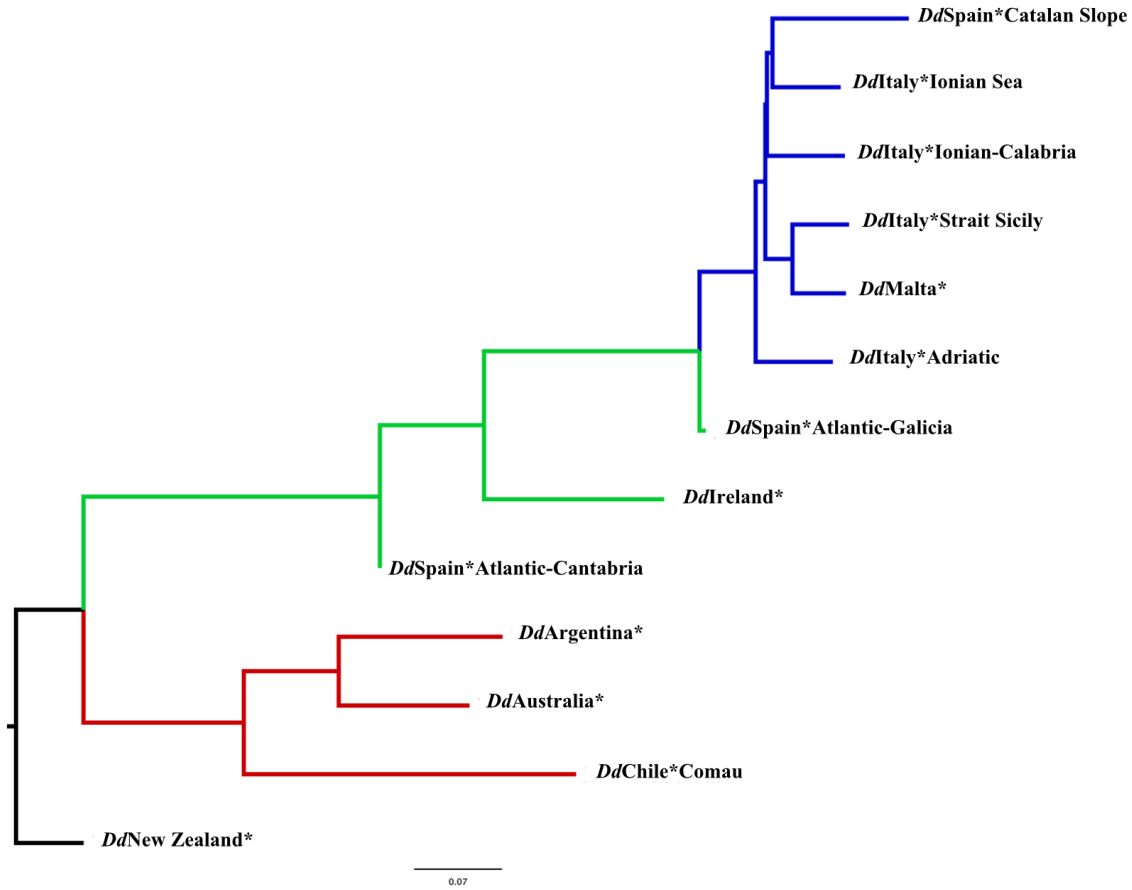

Figure 4.5. Phylogeography hypothesis among localities from southern hemisphere (red), North Atlantic (green) and Mediterranean Sea (blue) based on microsatellite data. Topology rooted using New Zealand locality. *Dd= Desmophyllum dianthus*.

Furthermore, positive and highly significant genetic-spatial correlation was detected from Mantel test (p-value= 0.001): genetic distance between pairs of original localities increased significantly with marine geographic distance, showing a genetic population structuring with isolation by distance pattern at wide scale (Fig. 4.6). Localities appeared clustered in two genetic divergence groups: one cluster, represented by Mediterranean Sea versus North Atlantic Ocean (marine distance range 0-5,000 km), showed a genetic distance range (0.008-0.1) larger than second cluster (0.05-0.18), represented by Argentina-Chile versus Mediterranean Sea-North Atlantic Ocean (marine distance range 8,000-17,000 km), and Australia-New Zealand vs Mediterranean Sea-North Atlantic Ocean (marine distance range >20,000 km), and where the geographic distance between southern populations is an average value of 6,000 km (Fig. 4.6).

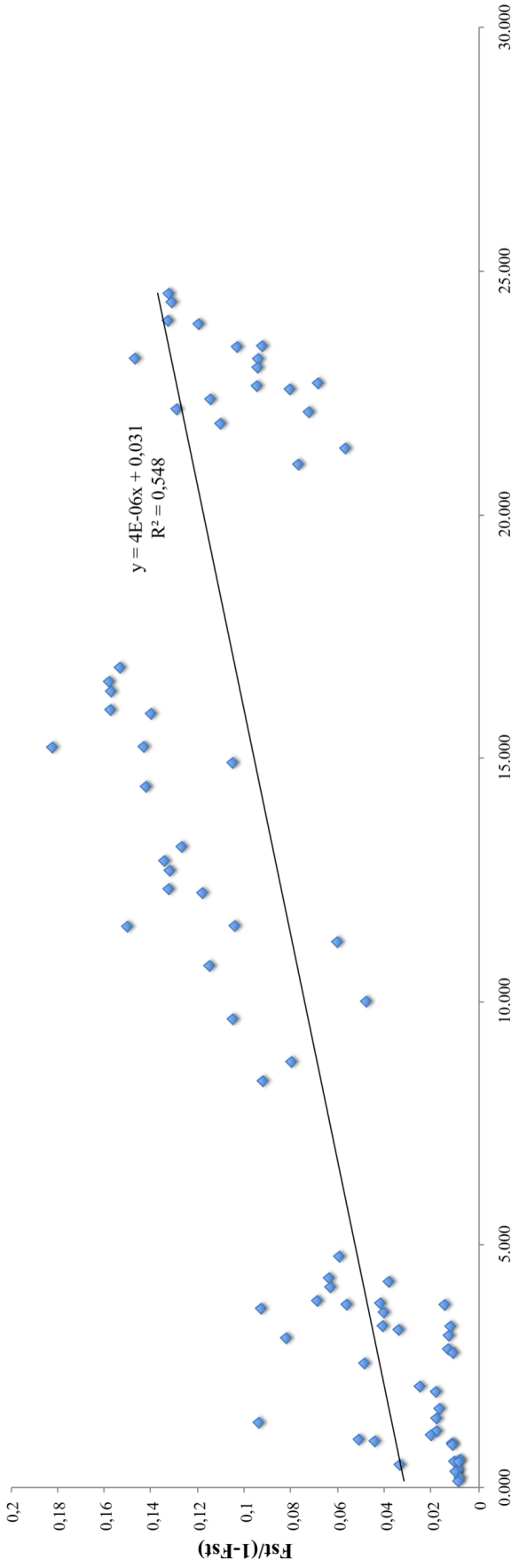

Figure 4.6. Mantel test for correlation between geographic and genetic distance among *Desmphyllum dianthus* localities.

Discussion

Deviations from HWE

Significant departures from the HWE proportions were found for 4 loci, whose observed genotype frequencies did not match with the frequencies expected for an ideal population (random mating, no mutation, no drift and no migration) (Selkoe and Toonen 2006), suggesting that sample analysed do not represent a panmictic population, or alternatively it might indicate the presence of null alleles. Null alleles can bias the results because they can create 'false-positive' departure from HWE. Mainly, three potential causes can lead to null alleles: 1) genotyping errors, in which PCR failure could be due to inconsistent or low DNA template quality (Gagneux *et al.* 1997; García de León *et al.* 1998), nucleotide sequence divergence in one or both flanking primers (Kwok *et al.* 1990) and differential amplification of size-variant alleles (Wattier *et al.* 1998); 2) evolutionary forces, in which genetic markers exhibiting locus-specific effects (outliers) and associated with biological factors, such as the Wahlund effect, inbreeding, selection or mutation at or near a microsatellite locus (Chakraborty *et al.* 1992), could also increase F_{ST} of selected or linked loci and cause deviations from HWE; and 3) demographic events (e.g. bottleneck), in which large variances in reproductive success (and recruitment) and/or the possibility of asexual reproduction (Baus *et al.* 2005; Strathmann *et al.* 1984) could shape particular genetic structure of population (see Dakin and Avise 2004 for review). Results from Micro-Checker, and after relative adjustments with Brookfield-1 principle for null alleles and sequential Holm-Bonferroni correction for multiple tests, showed that genotyping errors could be discarded as the main cause. Comparing results from three different methods used to detect selective loci, revealed inconsistent identifications of outliers from each other. Methodology for the outlier detection is based on presence of 'distinct' population differentiation coefficients (F_{ST}) from those under neutral expectations. This strategy has been widely used to detect recent episodes of selection in non-model species, where the absence of detailed genomic information does not allow other alternatives (Pérez-Figueroa *et al.* 2010). Microsatellites are characterized for their high mutation rate, which could lead to an underestimation of differentiation between populations affected by homoplasy, causing a slight overestimation in the proportion of outliers (Caballero *et al.* 2008). In a case of substantial homoplasy, mutation could give false positive, camouflaging a real

differentiation between populations. Although, allelic richness is relevant in a long-term perspective as selection limits, it is determined by the initial allelic composition more than by heterozygosity and maybe useful as an indication of a decrease in population size or past bottleneck (Leberg 2002; Foulley and Ollivier 2006). For these reasons and for the results obtained from outlier and linkage disequilibrium tests, loci B118, C102, DdL7, and DdL84 were not considered under selective pressure or linked genomic loci and were included into successive analyses, considering their departures from HWE might be attributed to demographic events or Wahlund effect, or asexual reproduction, and migration. However, further studies should be taken in consideration in order to investigate in-depth which events are responsible for observed deviations from HWE.

Given these considerations, inferences on population structure and population biology were made based on results obtained from analyses performed in this study, including all 30 loci.

Genetic structure

On a vast scale, strong genetic discontinuities were detected between populations inhabiting in northern and southern hemispheres, whose particular genetic characteristics are also highlighted. Even though the sample size of boreal population is twice than austral ones, the N_A observed is similar in both hemispheres, whereas the H_{OBS} in southern hemisphere is surprisingly less (homozigosy excess) than the one detected in the northern counterpart. In addition, P_A is much higher in the southern population (> 70%), suggesting that distinct ecological (biotic and abiotic factors) and demographic events (e.g. bottleneck or vicariance processes) have affected southern hemisphere more deeply or earlier. Although a clear isolation by distance pattern was detected indicating that gene flow is restricted among geographically distant populations, evidences of potential corridor between both hemispheres arise from North Atlantic populations (e.g. Cantabria and Ireland), whose genotyping assignments were quite equally distributed in both primary clusters. Therefore, due to discontinuity of sampling and heterogeneity of sampling size, larval dispersal among intermediate 'stepping-stone' populations between both hemispheres is not excluded. Moreover, the phylogeographic pattern showed differences between septentrional and meridional samples: in the north the relationships between sites are closer than those found in the

south. A good example is shown by the case of the Argentina-Chile and Galicia-Adriatic, whose samples are separated by almost the same marine geographic distance. In the cited southern sites, there is no sister group relationship between them (even if they are the geographically closest) and the branches lengths are greater than those from the North Atlantic and Central Mediterranean, indicating a greater or more ancient genetic isolation in the south.

On a broad scale, multiple processes (such as species mobility, divergence selection and oceanographic features) that influence gene flow and connectivity at different spatial scale could contribute to patterns of differentiation in *D. dianthus* in each ocean region. In South Pacific Ocean, strong genetic discontinuities between Australia, New Zealand and Chile were detected, indicating potential vicariance or regional adaptation events, as it was also shown in *Lophelia pertusa* in the North Atlantic Ocean (Morrison *et al.* 2011). Australia, New Zealand and Chile represent three distinct marine habitats, and *D. dianthus* specimens were sampled from a seamount, deep trench and shallow water fjord habitats respectively, thus genetic variation could be related to different oceanographic features characterized each habitats. Nevertheless, the unexpected connectivity between Australia and Argentina, whose samples were collected from a continental shelf, suggests that deep currents could play a key role in the delimitation of genetic barriers; in this case the gene flow could be explained by Antarctic circumpolar current (ACC) and its two principal fronts, the Subantarctic Front (SAF) and the Polar Front (PF). With the northward turn of the SAF east of Drake Passage, a thick layer of Circumpolar Deep Water (CDW) is noticed over the Falkland Plateau into the Argentine Basin. There it is joined by waters entering the Argentine Basin via a deep spreading route through the Georgia Basin: denser CDW, deep water from the Weddell Sea, and episodically, deep water from the southeastern Pacific Ocean (Peterson and Whitworth 1989). Therefore, results suggested isolation by depth rather than by distance.

At large scale, similar findings were observed for North East Atlantic Ocean and Mediterranean Sea, where genetic differentiation by geographic pattern is broken by unexpected low genetic flow between Cantabria and Galicia and high genetic similarity between Galicia and Mediterranean Sea. In the former case, inbreeding coefficients were substantial high for Galicia and Cantabria, and may be explained by restricted

gene flow between both marine regions, probably due to hydrographical and dynamic features of Cantabria and Galicia shelf areas or to small population size, hence characterized by heterozygosis deficit, or for presence of null alleles. Similar findings were reported in Le Goff-Vitry *et al.* (2004) and Morrison *et al.* (2011), but studies conducted looking into the characteristic of Gulf of Biscay revealed a high heterogeneous submarine orography (e.g. narrow canyon) and hydrology (e.g. seasonal upwelling system or eastward shelf–slope current, a prolongation of Iberian Poleward Current (IPC), etc.), that could contribute to determine a determinate larval distribution in western Iberia and Cantabrian Sea (Koutsikopoulos and Le Cann 1996; Sánchez and Gil 2000; Quinteiro *et al.* 2007; ICES 2008; Rivera *et al.* 2013). In the latter case, the unexpected connectivity between Galicia and Mediterranean Sea could be explained by Mediterranean Water Vein (MW), a poleward current that tends to contour the southwestern slope of Iberia, generating mesoscale features called Meddies, which can transport salty and warm MW over a great distance (Cherubin *et al.* 1997; Iorga Ciobotaru 1999; Paillet *et al.* 1999; ICES 2008). The MW effect is very clear in the western Spanish coast at the level of 1,200 m and a proportion of 77% MW is still found at this area, though it decreases at the northern cost of Galicia (Fraga *et al.* 1982). All these considerations led to reject Galicia as potential corridor between North Atlantic (Ireland and Cantabria) and Mediterranean Sea.

At small scale, several localities were sampled and analysed throughout the Mediterranean Sea, and most of them were located in the Central Mediterranean Sea. Intraspecific phylogenetic breaks and/or genetic transition that often occur for shallow-water benthic invertebrates (e.g. *Dendropoma petraeum*, Calvo *et al.* 2009), are usually associated with the marine biogeographic regions identified for the Mediterranean Sea (Bianchi and Morri 2000). Contrary to such geographical boundaries pattern, any substantial genetic differentiation was detected among *D. dianthus* individuals analysed from different Mediterranean localities. Exception made for Catalan Slope, which appeared slightly different from the Central Mediterranean but more similar to Galicia. Three hypotheses are considered: 1) present unidirectional gene flow: present gene connectivity could be explained by the Liguro-Provençal-Catalan Current System, characterized by Levantine Intermediate Water (LIW) and Western Mediterranean Deep water (WMDW), MW outflowing at Gibraltar (Cherubin *et al.* 1997; Millot and

Taupier-Letage 2005; Birol *et al.* 2010), allowing a drift of larvae to the Atlantic Ocean; 2) past unidirectional gene flow: genetic similarity could be related to the re-flooding of the Mediterranean Sea through the Zanclean or post-Messinian flood 5.33 million ago, allowing the marine biota from Atlantic Ocean to disperse freely into Mediterranean Sea. Successive periods of colonization and isolation left longitudinal gradients in genetic diversity across the Mediterranean and in some cases resulted in greatest genetic diversity in the central Mediterranean as a result of bidirectional colonization and secondary contact (Arnaud-Haond *et al.* 2007). Thus, slight genetic divergence of Catalan Slope individuals from the rest of individuals in the Mediterranean Sea could be attributed at a later stage to the Liguro-Provençal-Catalan Current System that differs from the Circulation System of Eastern Basin; 3) Mediterranean refugia: Catalan Slope and Central Mediterranean could have been two isolated potential refugia as well as potential centres of origins during the Messinian Salinity Crisis, thus evolving independently (Calvo *et al.* 2009). Vicariance between eastern and western Mediterranean population were proposed for other species using different genetic markers (Petit *et al.* 2003; Arnaud-Haond *et al.* 2007; Lowe *et al.* 2012; Triest and Sierens 2014).

Although low connectivity and declining genetic variability along a depth gradient was reported in previous studies for *D. dianthus* population in South Pacific Ocean - as well as in the Mediterranean Sea for *Corallium rubrum*, a deep-sea octocoral species, (Costantini *et al.* 2011; Miller *et al.* 2011) -, findings from this study suggest that it should be further tested at different scale and with a larger sample size.

Management implications

Inferring genetic variation at different spatial scale plays a key role in the conservation management. When the reproductive and development biology of a species is unknown, data on genetic variation and gene flow are relevant to infer biological or ecological events that could have affected the species. From a conservation point of view, making genetic data available is important, for example, in case a population is negatively affected and it is known that gene exchange exists between populations; then the loss of reef areas due to human activities or natural events, will not be damaging the overall genetic diversity of the species sites, which may be recolonized over time by sexually

produced larvae (Le Goff-Vitry *et al.* 2004). Regard to *D. dianthus* populations analysed in this study, the obtained results could be useful for any conservation measures plan necessary for the studied area. For example, in the case that a catastrophic event occurs within the Mediterranean population, our results suggest that Atlantic Ocean could play a role as genetic source allowing the recolonization (source-sink model) of the damage area, or vice-versa. On contrast, in the case of Chile or New Zealand populations, which resulted two different panmictic populations (so far), any negative event could dramatically damage them. Without any source for population recovery, the corresponding gene pools will be lost, affecting the genetic diversity of the species. Thus a especial conservation plans should be considered for singular populations as those of Chile and New Zealand, for example.

Although the overall relationship between genetic structure and marine life histories seems to generally match, there is a growing number of exceptions that provides powerful insights into the relationships between physical and biological oceanography (Palumbi 2004). Genetic population studies on a global scale allow having an overview on populations structure and connectivity, that provide the identification of potential genetic breaks and biogeographic boundaries in marine regions; also useful to accomplish a effective marine conservation. Furthermore, the scientific community should get in step with industry progress and work closely for a sustainable management of resources, even though it is not always possible. Therefore, a global view may help to prioritize some areas as 'hot spots' for further studies and/or conservation management plans.

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CHAPTER V

Chapter adapted from:

Addamo AM, Jermiin L, Gordon K, Taviani M, Machordom A (In prep.) Testing the strength of phylogenetic signal for old and new molecular markers, and their utility in coral phylogeny.

Desmophyllum vitreum. Charles Joseph Gravier (1920). Madreporaires provenant des Campagnes des yachts Princesse-Alice et Hirondelle II (1893-1913).

Testing the strength of phylogenetic signal for old and new molecular markers, and their utility in coral phylogeny.

Abstract

The use of integrative taxonomy, combining molecular and newly developed morphological characters, has a key role in the rearrangement of scleractinian systematics. Nevertheless, few studies have been focused on testing the strength of the phylogenetic signal of those markers and searching for new ones that could improve the understanding of scleractinian evolution. This study provides a phenetic and phylogenetic comparison between newly developed molecular markers and those frequently used, underlying issues about phylogeny and systematics of Scleractinia. The novel potential molecular markers and their phylogenetic signals are presented at different taxonomic level: family-genera-species.

Keywords: molecular markers, uncorrected p-distance, substitution saturation test, phylogeny reconstruction, Scleractinia, Systematics.

Introduction

The scleractinian taxonomic classification has been essentially based on the skeleton morphology, but its combination with molecular data provided new hypotheses for coral relationships and evolution (Budd *et al.* 2010; Zlatarski and Stake 2012). In the last decade, many studies have been published combining multiple molecular markers with morphological characters - including increasingly large number of corals species and applying an accurate data treatment -, providing new rearrangements at all taxonomic levels: family, genus, and species (Le Goff-Vitry *et al.* 2004; Medina *et al.* 2006; Fukami *et al.* 2008; Budd and Stolarski 2009; Barbeitos *et al.* 2010; Benzoni *et al.* 2010; Kitahara *et al.* 2010a; Kitahara *et al.* 2010b; Benzoni *et al.* 2011; Huang *et al.* 2011; Stolarski *et al.* 2011; Arrigoni *et al.* 2012; Benzoni *et al.* 2012; Budd *et al.* 2012; Kitano *et al.* 2013; Arrigoni *et al.* 2014a; Arrigoni *et al.* 2014b; Benzoni *et al.* 2014;

Huang *et al.* 2014a; Huang *et al.* 2014b; Kitano *et al.* 2014; Schmidt-Roach *et al.* 2014). However, the number of species placed as '*incertae sedis*' have not been diminished. Increasing the number of taxa could also perturb phylogenetic relationships, previously considered as stable as well as the starting point of the coral classification. This inconsistency is due to wrong classification based on morphological characters and/or selection of molecular markers used in the phylogeny study, leaving 'coral taxonomists and systematics to continue to be plagued by a host of problems' and works that still need to be done (Huang *et al.* 2009). So far, few studies were focused on testing the utility of molecular markers for the coral phylogeny, leading a lack of information on the strength of their phylogenetic signal (Jarman *et al.* 2002; Vollmer and Palumbi 2004; Shearer *et al.* 2005).

Since COI was recognized as an unsuccessful universal molecular barcoding system for several groups of species (Meyer and Paulay 2005; Huang *et al.* 2008; Shearer and Coffroth 2008; Bucklin *et al.* 2011; Krishna Krishnamurthy and Francis 2012), phylogeneticists have been searching new markers able to clarify the phylogenetic relationship at least into an entire order of organisms, and have so far been using a group of genes as 'barcoding molecular set' to reconstruct the phylogeny of the Tree of Life (see (Nosenko *et al.* 2013).

Given these considerations, the aim of this study is to search new molecular markers, testing their potential phylogenetic signal under phenetic and cladistics criteria, and finally comparing them with those markers currently used in coral phylogenetics. Preferential selection through all scleractinian corals was done for coding-protein genes that might be produced by single copy genes and homologous. Although, molecular phylogeneticists often believe that amino acid sequences would generate more reliable trees than nucleotide sequences, because third codon in protein-coding genes often evolve so fast that substantial saturation would occur between highly diverged taxonomic groups (Xia *et al.* 1996; Xia 1998; Xia *et al.* 2003). However, the higher variability of these nucleotide data brings useful characters to establish relationships between closely related organisms that might not be differentiated at the amino-acid level, for a small scale studies.

Material and Methods

Samples and DNA extractions

A series of molecular markers were developed to infer the phylogenetic relationship among scleractinian species at different taxonomic levels/resolution. Seven azooxanthellate coral species were selected for next generation sequencing: *Desmophyllum dianthus*, *Caryophyllia smithii*, *Paraconotrochus antarctica*, *Dendrophyllia ramea*, *Javania cailleti*, *Madrepora oculata* and *Oculina patagonica.* Ninety-four representatives of scleractinian species, 11 of which were investigated for the first time, were included in the following analyses. The specimens of coral were selected to represente 9 families and 46 genera in both 'complex' and 'robust' groups (Romano and Palumbi 1996), as well as to evaluate interspecific polymorphism and phylogenetic potential of novel markers. The list of the species used in this study can be found in Table 5.1. This study did not involve endangered or protected species listed in the IUCN Red List of Threatened Species. All necessary campaigns permits were obtained for the described field studies.

For high throughput sequencing, genomic DNA (gDNA) was extracted from an entire polyp in order to obtain a final DNA concentration of 2.5 μg/μl, using the QIAGEN BioSprint 15 DNA Blood Kit (Qiagen Iberia S.L., Madrid), with slight modifications, including the optional RNase treatment and an extended period of proteinase K lysis (overnight incubation at 55 °C). DNA concentration was quantified using the Qubit 2.0 Fluorometer. For subsequent amplifications gDNA was extracted as above, even though from mesenteric tissue and diluted to a final concentration of 2 ng/μl.

Table 5.1. List of corals included in the analyses. Information of success (+) or failed (-) PCR amplification

Family	Genus	*Spp.*	Country (Province/State, Locality)	Original Code	Repository (Institute)	PCR
Acroporidae	*Acropora*	*hemprichii*	Yemen (Gulf of Aden, Bir Ali)	BA071	UNIMIB	+
Acroporidae	*Acropora*	*hyacynthus*	Yemen (Gulf of Aden, Bir Ali)	BA091	UNIMIB	+
Acroporidae	*Acropora*	*valida*	Yemen (Gulf of Aden, Bir Ali)	BA130	UNIMIB	+
Caryophylliidae	*Anomocora*	*fecunda*	Portugal (Madeira Islands)	100252	USNM- NMNH	-
Caryophylliidae	*Aulocyathus*	*atlanticus*	Marroco	MNHN-IK-2011-2458	MNHN	-
Caryophylliidae	*Caryophyllia (Caryophyllia)*	*calveri*	Italy (off Santa Maria di Leuca)	AMA-282	MNCN	+
Caryophylliidae	*Caryophyllia (Caryophyllia)*	*cornuformis*	United States	1010389	USNM- NMNH	+
Caryophylliidae	*Caryophyllia (Caryophyllia)*	*cyathus*		MNHN-IK-2011-2288	MNHN	+
Caryophylliidae	*Caryophyllia (Caryophyllia)*	*diomedeae*	United States	1072329	USNM- NMNH	+
Caryophylliidae	*Caryophyllia (Caryophyllia)*	*huinayensis*	Chile (Patagonia, Pitipalena Fjord)	AMA-58	MNCN	+
Caryophylliidae	*Caryophyllia (Caryophyllia)*	*seguenzae*	Spain (Aviles Canyon)	004-V03	IEO- Santander	+
Caryophylliidae	*Caryophyllia (Caryophyllia)*	*smithii*	Spain	AMA-40	MNCN	+
Caryophylliidae	*Ceratotrochus*	*magnaghii*	France (Marseille, Cave Rion)	98482	USNM- NMNH	+
Caryophylliidae	*Conotrochus*	*funicolumna*	Wallis and Futuna Islands	98722	USNM- NMNH	-
Caryophylliidae	*Desmophyllum*	*dianthus*	Chile (Patagonia, Pitipalena Fjord)	DdIJC432	MNCN	+
Caryophylliidae	*Desmophyllum*	*dianthus*	Italy (off Rocella Ionica)	DdROC163	MNCN	+
Caryophylliidae	*Labyrinthocyathus*	*facetus*	United States	1114920	USNM- NMNH	+
Caryophylliidae	*Lophelia*	*pertusa*	Italy (off Santa Maria di Leuca)	AMA-52	MNCN	+
Caryophylliidae	*Lophelia*	*pertusa*	Ireland (Moira Mounds)	AMA-296	MNCN	+
Caryophylliidae	*Paraconotrochus*	*antarctica*	SubAntarctic	AMA-44	MNCN	+
Caryophylliidae	*Polycyathus*	*senegalensis*	United States (Texas, West Flower Garden Bank)	1026497	USNM- NMNH	+
Caryophylliidae	*Pourtalosmilia*	*anthophyllites*	Spain (Algeciras)	AMA-37	MNCN	+
Caryophylliidae	*Solenosmilia*	*variabilis*	Argentina (Patagonia)	PATA 10/08 DR13	IEO- Gijón	-
Caryophylliidae	*Stephanocyathus*	*crassus*		007-G15	IEO- Santander	-
Caryophylliidae	*Stephanocyathus*	*diadema*	United States	100906	USNM- NMNH	+
Caryophylliidae	*Stephanocyathus*	*moseleyanus*	Spain (Aviles Canyon)	001-V03	IEO- Santander	+
Caryophylliidae	*Stephanocyathus*	*nobilis*	Spain (Aviles Canyon)	012-V10	IEO- Santander	-
Caryophylliidae	*Stephanocyathus*	*platypus*	New Zealand	94164	USNM- NMNH	-
Caryophylliidae	*Stephanocyathus*	*regius*	Vanuatu	98655	USNM- NMNH	+
Caryophylliidae	*Stephanocyathus (Acinocyathus)*	*spiniger*	Philippines (Mindoro Island)	97143	USNM- NMNH	-
Caryophylliidae	*Stephanocyathus (Odontocyathus)*	*coronatus*	Fiji	1100225	USNM- NMNH	+
Caryophylliidae	*Stephanocyathus (Odontocyathus)*	*weberianus*	Vanuatu (Espiritu Santo Island)	98662	USNM- NMNH	-
Caryophylliidae	*Tethocyathus*	*endesa*	Chile (Patagonia, Pitipalena Fjord)	AMA-59	MNCN	+
Caryophylliidae	*Trochocyathus*	*aithoseptatum*	United States	1072318	USNM- NMNH	+
Caryophylliidae	*Trochocyathus*	*philippinensis*	Vanuatu (Tanna Island)	98638	USNM- NMNH	-
Caryophylliidae	*Vaughanella*	*concinna*	Spain (Aviles Canyon)	038-DR15	IEO- Santander	+
Caryophylliidae	*Vaughanella*	*margaritata*		1008600	USNM- NMNH	+
Deltocyathiidae	*Deltocyathus*	*magnificus*	Indonesia	MNHN-IK-2011-2385	MNHN	-
Deltocyathiidae	*Deltocyathus*	*suluensis*	Vanuatu	98670	USNM- NMNH	-
Dendrophylliidae	*Astroides*	*calycularis*	Spain	AMA-56	MNCN	+
Dendrophylliidae	*Balanophyllia*	*europea*	Spain (Almeria, La Isleta del Moro)	AMA-48	MNCN	+
Dendrophylliidae	*Balanophyllia*	*regia*	Spain (Almeria, La Isleta del Moro)	AMA-47	MNCN	+
Dendrophylliidae	*Cladopsammia*	*echinata*	Indonesia (Selaru Isalnd)	97628	USNM- NMNH	+
Dendrophylliidae	*Dendrophyllia*	*alcocki*	Palau	1006512	USNM- NMNH	+
Dendrophylliidae	*Dendrophyllia*	*arbuscula*	Indonesia	97632	USNM- NMNH	-
Dendrophylliidae	*Dendrophyllia*	*cornigera*	France	98475	USNM- NMNH	-
Dendrophylliidae	*Dendrophyllia*	*ijimai*	Indonesia	MNHN-IK-2011-2398	MNHN	-
Dendrophylliidae	*Dendrophyllia*	*johnsoni*	Ecuador	98444	USNM- NMNH	+
Dendrophylliidae	*Dendrophyllia*	*laboreli*	Spain	AMA-39	MNCN	+
Dendrophylliidae	*Dendrophyllia*	*oldroydae*	Ecuador	84845	USNM- NMNH	-
Dendrophylliidae	*Dendrophyllia*	*ramea*	Spain (Cadiz)	AMA-45	MNCN	+
Dendrophylliidae	*Eguchipsammia*	*serpentina*	United States (Hawaii, Kure Island Bank)	1072335	USNM- NMNH	-

Table 5.1 (continued). List of corals included in the analyses. Information of success (+) or failed (-) PCR amplification

Family	Genus	*Spp.*	Country (Province/State, Locality)	Original Code	Repository (Institute)	PCR
Dendrophylliidae	*Endopachys*	*grayi*	Somalia (off Cape Guardafui)	98983	USNM- NMNH	+
Dendrophylliidae	*Rhizopsammia*	*wettsteini*	Yemen (Gulf of Aden, Balhalf)	Y756	UNIMIB	+
Dendrophylliidae	*Thecopsammia*	*socialis*	United States (NC, Cape Fear)	1114650	USNM- NMNH	+
Dendrophylliidae	*Tubastraea*	*aurea*	Yemen (Gulf of Aden, Balhalf)	Y755	UNIMIB	+
Dendrophylliidae	*Tubastraea*	*micranthus*	Yemen (Gulf of Aden, Balhalf)	Y757	UNIMIB	+
Flabellidae	(Flabellum?)	*unidentified*		MNHN-IK-2011-2461	MNHN	-
Flabellidae	*Flabellum*	*alabastrum*	United States (Massachussetts, Georges Bank)	1008601	USNM- NMNH	+
Flabellidae	*Flabellum*	*alabastrum*		025-DR15	IEO- Santander	+
Flabellidae	*Flabellum*	*alabastrum*		004-G09	IEO- Santander	-
Flabellidae	*Flabellum*	*angulare*	United States	1083899	USNM- NMNH	-
Flabellidae	*Flabellum*	*apertum apertum*	New Zealand	94298	USNM- NMNH	+
Flabellidae	*Flabellum*	*curvatum*	Argentina (Patagonia)	PATA08	IEO- Gijón	+
Flabellidae	*Flabellum*	*flexuosum*		82188	USNM- NMNH	+
Flabellidae	*Flabellum*	*impensum*		MNHN-IK-2011-2437	MNHN	+
Flabellidae	*Flabellum*	*knoxi*	New Zealand	94332	USNM- NMNH	+
Flabellidae	*Flabellum*	*lamellulosum*	Philippines	MNHN-IK-2011-2386	MNHN	+
Flabellidae	*Flabellum*	*lowekeyesi*	New Zealand	94318	USNM- NMNH	+
Flabellidae	*Flabellum*	*merrum*	Indonesia	MNHN-IK-2011-2513	MNHN	-
Flabellidae	*Flabellum*	*moseleyi*	United States	76409	USNM- NMNH	-
Flabellidae	*Flabellum*	*sp.new*	Argentina (Patagonia)	PATA02	IEO- Gijón	+
Flabellidae	*Flabellum*	*thouarsii*	Argentina (Patagonia)	PATA06	IEO- Gijón	+
Flabellidae	*Flabellum (Ulocyathus)*	*deludens*		96669	USNM- NMNH	-
Flabellidae	*Javania*	*antarctica*	Argentina (Patagonia)	PATA03	IEO- Gijón	+
Flabellidae	*Javania*	*borealis*		1011112	USNM- NMNH	+
Flabellidae	*Javania*	*cailleti*	Spain (Galicia Bank)	026-DR15	IEO- Santander	+
Flabellidae	*Javania*	*exserta*	Palau	100155	USNM- NMNH	-
Flabellidae	*Javania*	*insignis*	Philippines	MNHN-IK-2011-2420	MNHN	-
Flabellidae	*Javania*	*lamprotichum*		1071211	USNM- NMNH	+
Flabellidae	*Monomyces*	*rubrum nobile*	New Zealand (Cape Maria Van Diemen)	94342	USNM- NMNH	-
Flabellidae	*Polymyces*	*wellsi*	United States (Hawaii, Pioneer Bank)	1072331	USNM- NMNH	+
Flabellidae	*Truncatoflabellum*	*mortensoni*	Vanuatu	98904	USNM- NMNH	-
Flabellidae	*Truncatoflabellum*	*paripavoninum*	Philippines (Mindoro Island)	97548	USNM- NMNH	-
Fungiacyathidae	*Fungiacyathus*	*stephanus*	Indonesia	MNHN-IK-2011-2388	MNHN	+
Fungiacyathidae	*Fungiacyathus*	*variegatus*	Philippines	MNHN-IK-2011-2412	MNHN	-
Incertae sedis	*Cladocora*	*caespitosa*	Spain (Columbretes, Puerto Tofiño)	AMA-286	MNCN	+
Merulinidae	*Dipsastraea*	*matthai*	Yemen (Gulf of Aden, Bir Ali)	BA101	UNIMIB	+
Merulinidae	*Dipsastraea*	*pallida*	Yemen (Gulf of Aden, Bir Ali)	BA061	UNIMIB	+
Merulinidae	*Dipsastraea*	*pallida*	Yemen (Gulf of Aden, Bir Ali)	BA119	UNIMIB	+
Micrabaciidae	*Leptopenus*	*discus*	United States (California, Fielberling Guyot)	93938	USNM- NMNH	-
Micrabaciidae	*Rhombopsammia*	*niphada*	Indonesia (Moluccas, Sera Island)	96737	USNM- NMNH	+
Oculinidae	*Bathelia*	*candida*	Argentina (Patagonia)	PATA 11/08 DR04	IEO- Gijón	-
Oculinidae	*Cyathelia*	*axillianis*	Philippines	MNHN-IK-2011-2495	MNHN	-
Oculinidae	*Madrepora*	*oculata*	Italy	AMA-51	MNCN	+
Oculinidae	*Oculina*	*patagonica*	Spain	AMA-50	MNCN	+
Pocilloporidae	*Madracis*	*pharensis*	Libanon	MNHN-IK-2011-2472	MNHN	-
Turbinoliidae	*Tropidocyathus*	*lessoni*	Philippines	MNHN-IK-2011-2384	MNHN	-
Gorgoniidae	*Letpogorgia*	*pulchra*	Ecuador	M111	MNCN	+
Stylasteridae	*Pliobathrus*	*symmetricus*	Spain	MNHN-IK-2011-2243	MNHN	+

IEO	Instituto Español de Oceanografía (Gijon, Spain) (Santander, Spain)
MNCN	Museo Nacional de Ciencia Naturales (Madrid, Spain)
MNHN	Muséum National d'Histoire Naturelle (Paris, France)
UNIMIB	University of Milano-Bicocca (Milan, Italy)
USNM- NMNH	National Muesum of Natural History (Washington DC, USA)

Genome shotgun sequencing

The genomic library was constructed at the Genomics Research & Services (Parque Cientifico de Madrid, Biomol-Informatics SL, Cantoblanco, Madrid, Spain). Two point five micrograms of gDNA of each specimen were fragmented using a Bioruptor (Diagenode). DNA fragments size around 400 bp were excised from agarose gels and purified, and TruSeq libraries (Illumina) were prepared according to the manufacturer's instructions. DNA libraries were checked for size, concentration, and integrity using Bioanalyzer (Agilent) and quantified by quantitative PCR (qPCR) in order to accurately estimate the quantity of DNA. Genome shotgun sequencing was performed using an Illumina GAIIx sequencer and each library was sequenced in seven separated lines. Paired-end reads (2x100) were generated according to the manufacturer's instructions (Illumina, Inc.). Single reads of 100 nucleotides were obtained and raw reads were subjected to quality-filtered (removal of adapters, artefacts, low-quality reads and duplicates) using the standard Illumina process and analysed using FastQC tool (Andrews). An average of $66x10^6$ reads per species were used for *de novo* assembly using SOAPdenovo v.1.05 package (Luo *et al.* 2012) (Table 5.2) and oriented into scaffolds using Mugsy open-source software (Angiuoli and Salzberg 2010). The identification of orthologous groups and the genome annotation were performed using the OrthoMCL (Li *et al.* 2003) and SWISS-PROT (Bairoch and Apweiler 1996), for multiple sequence alignment of the seven coral species.

Table 5.2. GaIIx Information and number of scaffolds and multiple alignment per each species. M= mega; %Cover= percentage of coverage of sequencing. *Calculated considering genome size of *Acropora digitifera* (420 Mbp).

Species	Insertion (Average)	Reads	Size M	% Cover*	No. Scaffolds	No. Alignment
Desmophyllum dianthus	430	64208496	64	15,24	194379	39916
Caryophyllia smithii	513	57495676	57	13,57	221391	40894
Paraconotrochus antarctica	524	56254806	56	13,33	142013	26151
Dendrophyllia ramea	470	76154662	76	18,10	277008	24513
Javania cailleti	541	96834006	99	23,57	191043	24637
Madrepora oculata	453	52968500	53	12,62	26214	10067
Oculina patagonica	511	56996784	57	13,57	269768	27105

New markers discovery

To isolate new markers for phylogenetic analysis, more than $1x10^6$ scaffolds were manually filtered by length (set to ≥ 500 bp) and identity (set to ≥ 70%); also a sequence consensus of multiple copies was considered. Multiple species alignment of the selected

scaffolds were manually filtered by length (set to ≥ 200 bp), strand representation and interspecific polymorphism using Gmaj (Blanchette *et al.* 2004). A total of 50 multiple alignments found were then manually checked with Seaview 4.5.0 (Gouy *et al.* 2010); 26 alignments were finally selected by length (set to ≥ 400 bp), variable sites (set to ≥ 2%) and informative sites (set to ≥ 10%).

Primers design and testing

Degenerate primers pairs were designed and compared using CODEHOP (Rose *et al.* 2003) and HYDEN (Linhart and Shamir 2007). Primers designations were manually checked in order to reduce degeneracy by using the ability of 'mismatched' base pairs, like guanine-thymine, to form a partial bond in primer-template interactions (Palumbi 1996). Corresponding amino acid translation of primers pairs were manually checked in order to maximize efficiency of universal primer pairs by stretching the primers match over 7-9 identical amino acid (Palumbi 1996). The corresponding genes for which primers pairs were developed are represented in Table 5.3.

Table 5.3. Information of primers for selected genes

Gene	Primer Forward 5'-3'	Primer Reverse 5'-3'
COR2-NAD3	CAYTCTARNCCCYCCTYTTARTC	CAATNGCAGCRGCNGAGTCTTC
COR3-AMPt1	TRCCYTCRACRGCATTRGAATGR	CTTAAACTNGCTYTGGANATG
COR4-AMPt2	GTTGAAACAAGNATGGCNGT	GTCCATTGGCRTCAGTRAAT
COR6-SIAH1	TCNGCTTGTTTACGTGTTCCAAT	ATGAATCGCCAANCAAGYTC
COR7-Actin	TCAACTGYCCAGCYATGTAYG	CAGGNAGCTCRTAGCTCTTCTC
COR10-ßActin	CAGATYATGTTCGAGACYTTCCA	RAANAGTGCTTCNGGRCATC
COR12-Helicase	CCAATGTTACCRGCTTGTTTRGTTT	TGCTGAGAAAAARGCTGATGTNGAT
COR14-NAD5	TTTYCTTCARTTRTTTATTGGNTG	CCCTAAAACYTTTCGTTCTGC
COR15-Creatine kinase	GTGCAGAARCGCTCGAANAC	CGYTRGACTCNCTNGATGGCG
COR17-NCAH-like	TGGGNAARCAAAACAGTAARCTCAA	GAACTTGACGCATCRCAYTG
COR21-UBB	CTAAAAATAGCNCATTATGAATTG	GRGTRGACTCYTTCTGGAT
COR24-Heat shock like	ATCCACTTCYTCAATAGTAGGTCCA	GTCCAAGGANGACATAGA
COR25-16S rDNA	GTACTGTGAAGGAAAGTTGAAAGAG	TGAYACCATTCATACCGGYCAA
COR26-ATP6NAD4	AAGCRCGAACYTTTTCTTCYC	GTNTNTTTGAATGTGYTGGG

Twenty-six primers pairs were initially used to test for successful PCR amplification and sequencing, by visualizing amplified products on 1.5% agarose gels and sequence chromatogram in Sequencher v4.10.1 (Gene-Code Corporation) in the seven selected coral species in order to confirm that selected loci were not potential chimera sequences due to *de novo* assembly. PCRs were carried out in a total volume of 50 µl with 1x PCR Biotools Standard Reaction Buffer including 2 mM $MgCl_2$, 0.5 mM forward and reverse primers, 0.2 mM of each dNTP, 1.5U DNA polymerase (Biotools), and 2 ng of template

DNA. PCR amplifications were performed in a Veriti™ Thermal Cycler (Applied Biosystems) with a ramped cycles profile (Palumbi 1991) with optimized modifications: an initial denaturing step of 94 ºC for 5 min, followed by 40 cycles of 30 s at 94 ºC, an initial annealing step of 10 s at 48 ºC, a 2 min ramp from 48 ºC to 72 ºC, and a final extension of 10 min at 72 ºC. When this profile did not get any amplification of a specific PCR product, we tested three other annealing and ramp temperatures ($T_{A/R}$ 45, 50, 52 or 56 ºC) with the same cycling conditions.

Testing the utility of novel molecular markers

Sequences were automatically aligned using ClustalX (Thompson *et al.* 1997) and subsequently, the resulted alignments were checked manually using Se-Al v2.0a11 (Rambaut 2002). To estimate the genetic divergence between pairs of taxa, uncorrected p-distances and neighbour joining searches (NJ analyses not shown) were calculated in PAUP*v4.0a134 (Swofford 2002). To calculate mean genetic distance among genera and families of corals, uncorrected p-distance was implemented in Sequencer 6.1 (shareware written by B. Kessing and available at: http://nmg.si.edu/sequencer/). To examine the degree of substitution saturation within the individual codon positions, all substitutions, transitions (Ti) and transversions (Tv) calculations were performed using PAUP*v4.0a134 (Swofford 2002), and represented in Excel files. To produce phylogenetic estimates from individual loci and to compare their contribution with the phylogenetic evidence, a set of analyses based on Maximum Parsimony (MP) and Bayesian Inference (BI) criteria was performed for the different data set using PAUP*v4.0a134 (Swofford 2002) for Maximum Parsimony (MP) and MrBayes v3.2.1 (Ronquist and Huelsenbeck 2003) for the Bayesian Inference (BI). The MP analyses were executed through a branch and bound search using a tree bisection and reconnection (TBR) algorithm and also included ten random stepwise additions. Bootstrap analysis (Felsenstein 1985) was used to infer the relative robustness tree branches (1000 pseudoreplicates). For the BI analyses, double parallels runs were performed for 5 millions of generations with one cold and three heated Markov Chains Monte Carlo (MCMC) for each run, sampling trees at 1000 generations intervals (5000 trees were saved during MCMC for each run), when the average standard deviation of split frequencies between runs was less than 0.01. To root the trees as well as to compare divergences within the Order Scleractinia, *Nematostella vectensis* and several

representatives of different genera and families of corals were taken from GenBank and have been included in the analyses (Annexe 2).

Genes commonly used in phylogenetic analyses

In order to extend as well as compare genetic divergences and phylogenetic signal between old and new molecular markers, four mitochondrial and three nuclear makers available for 31 scleractinian families, 176 genera and 560 species were obtained from GenBank, totalling around 3900 sequences (Annexe 2). These DNA genes include nuclear and mitochondrial markers with different mutation rates: 1) mitochondrial small subunit ribosomal RNA (12S), 2) mitochondrial large subunit ribosomal RNA (16S), 3) mitochondrial cytochrome c oxidase subunit I (COI), 4) mitochondrial cytochrome b (CYTB), 5) nuclear small ribosomal RNA subunit (18S), 6) nuclear large ribosomal RNA subunit (28S), and 7) the internal transcribed spacer regions (internal transcribed spacer 1-5.8S ribosomal DNA - internal transcribed spacer 2, hereafter designated ITS). Additionally, four (ITS, 28S, 16S, COI) of seven DNA genes and regions were newly amplified, sequenced and analysed for the first time for *Dendrophyllia laboreli* and *P. antarctica* following the same conditions performed in a previous study (Addamo *et al.* 2012). Matrices were aligned in ClustalX v.2 (Larkin *et al.* 2007) using default setting. The resulting alignments were manually checked and analysed as described above.

Results

Novel markers

Of the 26 potential loci markers tested, 8 were excluded due to PCR failure or multiple bands profiles and the remaining 18 ones were tested for their ability to amplify in a wide range of scleractinian species. In most cases successful PCR products have been obtained for each primer set, except for COR8, COR9, COR13, and COR16, in which no amplifications have been produced or where constant multiple products indicated a non-specific amplification of the gene. The remaining 14 genes for which further analyses were performed, were as follows:

COR2-NAD3

This is a protein-coding gene for **NADH-ubiquinone oxidoreductase chain 3.** This protein is a core subunit of the mitochondrial membrane respiratory chain NADH dehydrogenase (Complex I) that is believed to belong to the minimal assembly required for catalysis. Complex I functions in the transfer of electrons from NADH to the respiratory chain. The immediate electron acceptor for the enzyme is believed to be ubiquinone (source Uniprot.org). The total length of final alignment is 461 bp.

COR3-AMPt1

This is a protein-coding gene for **Adenosine monophosphate-protein transferase**. This protein mediates the addition of adenosine 5'-monophosphate (AMP) to specific residues of target proteins. Adenylyltransferase activity is inhibited by the inhibitory helix present at the N-terminus: Glu-204 binds ATP and competes with ATP-binding at Arg-344, thereby preventing adenylyltransferase activity. Activation dissociates ATP-binding from Glu-204, allowing an ordered binding of the entire ATP moiety with the alpha-phosphate in an orientation that is productive for accepting an incoming target hydroxyl side chain (source Uniprot.org). The total length of final alignment is 332 bp.

COR4-AMPt2

This is a protein-coding gene for **Adenosine monophosphate-protein transferase (**see previous gene for description). The total length of final alignment is 333 bp.

COR6-SIAH1

This is a protein-coding gene for **E3 ubiquitin-protein ligase**. This protein mediates ubiquitination and subsequent proteasomal degradation of target proteins. E3 ubiquitin ligase accepts ubiquitin from an E2 ubiquitin-conjugating enzyme in the form of a thioester and then it transfers directly the ubiquitin to the targeted substrates (source Uniprot.org). The total length of final alignment is 601 bp.

COR7-Actin

This is a protein-coding gene for **Actin**. Actin is the most abundant protein in most eukaryotic cells. It is highly conserved and participates in more protein-protein interactions than any other known protein. These properties make Actin a critical player

in many cellular functions, ranging from cell motility and maintenance of cell shape and polarity to the regulation of transcription (Dominguez and Holmes 2011). The total length of final alignment is 303 bp.

COR10-ßActin

This is a protein-coding gene for **ßActin** (see previous gene for description). The total length of final alignment is 383 bp.

COR12-Helicase

This is a protein-coding gene for **ATP-dependent RNA helicase**. This protein belongs to DEAD box helicase family. Helicases are ATPases that catalyze the unwinding of double-stranded nucleic acids. They are tightly integrated (or coupled) components of various macromolecular complexes, which are involved in processes such as DNA replication, recombination and nucleotide excision repair, as well as RNA transcription and splicing (source Uniprot.org). The total length of final alignment is 466 bp.

COR14-NAD5

This is a protein-coding gene for **NADH-ubiquinone oxidoreductase chain 5** (see previous gene COR2-NAD3 for description). The total length of final alignment is 779 bp.

COR15-Creatine kinase

This is a protein-coding gene for **Creatine kinase**. This protein belongs to the ATP: guanido phosphotransferase family. It reversibly catalyzes the transfer of phosphate between ATP and various phosphogens (e.g. creatine phosphate). Creatine kinase isoenzymes play a central role in energy transduction in tissues with large, fluctuating energy demands - such as skeletal muscle, heart, brain and spermatozoa (source Uniprot.org)-. The total length of final alignment is 252 bp.

COR17-NCAH-like

This is a protein-coding gene for **Neurocalcin like protein**. This protein binds at least one calcium atom, or one whose function is calcium-dependent. Calcium is essential for

a variety of bodily functions such as neurotransmission, muscle contraction and proper heart function (source Uniprot.org). The total length of final alignment is 528 bp.

COR21-UBB

This is a protein-coding gene for **Polyubiquitin**. Ubiquitin is a highly conserved 76-amino acid polypeptide that is found throughout the eukaryotic kingdom. The covalent conjugation of ubiquitin (often in the form of a polymer) to substrates governs a variety of biological processes ranging from proteolysis to DNA damage tolerance. The functional flexibility of this post-translational modification has its roots in the existence of a large number of ubiquitinating enzymes that catalyze the formation of distinct ubiquitin polymers, which in turn encode different signals (Li and Ye 2008). The PCR total length of final alignment is 730 bp.

COR24-Heat shock like

This is a protein-coding gene for **Heat shock like protein**. This protein belongs to the heat shock family and involves, during in the response to stress, a change in state or activity of a cell or an organism (in terms of movement, secretion, enzyme production, gene expression, etc.) as a result of some stressful conditions. The stress is usually, but not necessarily, exogenous (e.g. temperature, humidity, ionizing radiation, hypertonicity, amino acid deprivation) (source Uniprot.org). The total length of final alignment is 523 bp.

COR25-16S rDNA

This gene codifies for **mitochondrial large subunit ribosomal RNA**. 16S ribosomal RNA has a structural role, acting as a scaffold defining the positions of the ribosomal proteins and stabilizing correct codon-anticodon. 16S rRNA contacts alone are sufficient to support protein synthesis in living cell (Noller *et al.* 2005). The total length of final alignment is 1314 bp. This portion of 16S differs from the one commonly used in phylogeny studies of Scleractinia, and it is located around 1300 bp closer to position 5'.

COR26-ATP6NAD4

This sequence is formed by two fragments of protein-coding gene for **ATP synthase subunit a** and **NADH-ubiquinone oxidoreductase chain 4**. The former is a multi-pass membrane protein with hydrogen ion transmembrane transporter activity (source Uniprot.org). The latter belongs the complex I subunit 4 family (see previous gene COR2-NAD3 for description). The total length of final alignment is 1223 bp.

Genetic divergence and strength of phylogenetic signal

All the ranges of the average uncorrected p-distance obtained between families, genera and species overlapped each other for their minimum value, showing no genetic divergence between pairs of components of each group (e.g., Euphyliidae vs Meruliniidae, at family level; *Desmophylllum* vs *Lophelia*, at genus level). There were some exceptions, as those found among families analysed with COR24, COR21, COR17, COR12, and COR10 where the average of minimum p-distance started at 1.26%, 12.77%, 3.71%, 4.39%, and 1.39% respectively (Table 5.4, Figure 5.1a-c). In the range of maximum data values there were also noticeable figures, since values as high as 80% of divergence were found (Table 5.4). These huge values are related, in most cases, to sequences that overlapped in only a small part of their length. The lack of relationships among the different mean values found in the three levels analysed (families-genera-species) was also clear, as it is shown in Figure 5.2, probably due to bad taxonomic determinations or classifications in most of the cases, but the saturation could have also played a role.

Table 5.4. Uncorrected p-distance average, standard deviation (SD) and range between/within families and genera Min= minimum value; Max= maximum value.

	Between families				**Between genera**				**Between species**			
Gene	Average	SD	Min	Max	Average	SD	Min	Max	Average	SD	Min	Max
12S	14,91	7,11	0,00	29,01	2,59	4,86	0,00	22,54	0,79	1,85	0,00	19,63
16S	12,44	8,25	0,00	80,81	2,88	4,64	0,00	57,93	1,53	3,79	0,00	57,93
18SITS	17,52	7,49	0,00	70,27	5,66	7,74	0,00	65,19	2,27	4,20	0,00	65,19
28S	13,43	6,21	0,00	55,63	6,49	5,52	0,00	54,11	2,78	3,97	0,00	51,75
COI	14,13	6,74	0,00	60,66	1,58	2,23	0,00	20,26	0,59	1,57	0,00	18,69
COR2-NAD3	22,10	7,57	0,25	32,50	6,17	8,21	0,00	27,51	5,54	10,72	0,00	27,38
COR3-AMPt1	14,78	6,62	0,00	36,45	8,48	5,89	0,00	17,55	5,97	6,63	0,00	15,98
COR4-AMPt2	14,48	6,70	0,00	30,45	9,37	6,71	0,00	20,52	7,86	7,15	0,00	18,62
COR6-SIAH1	11,21	6,53	0,00	32,95	4,89	4,12	0,00	28,23	3,72	6,24	0,00	28,23
COR7-Actin	8,26	5,30	0,00	29,75	4,43	4,06	0,00	25,12	2,62	2,10	0,00	7,46
COR10-ßActin	18,51	9,00	1,39	34,02	16,45	10,52	0,26	28,68	7,27	11,77	0,26	28,57
COR12-Helicase	12,50	6,87	4,39	28,53	3,71	1,93	0,00	7,08	0,82	1,70	0,00	4,30
COR14-NAD5	19,77	10,93	0,21	36,82	6,60	10,26	0,00	35,37	1,82	7,38	0,00	35,37
COR15-Creatine kinase	13,22	5,88	0,83	23,45	7,85	5,31	0,00	17,44	5,67	4,72	0,00	13,86
COR17-NCAH-like	18,10	7,47	3,71	30,05	4,40	3,20	0,00	10,06	1,71	1,02	0,19	3,07
COR21-UBB	19,40	4,96	12,77	25,28	8,48	3,45	0,66	13,62	1,63	0,00	1,63	1,63
COR24-Heat shock like	19,20	6,61	1,26	33,60	4,86	4,83	0,00	18,19	0,99	1,04	0,00	2,31
COR25-16S rDNA	22,44	11,01	0,09	34,73	10,55	13,36	0,00	32,67	5,38	11,64	0,00	32,65
COR26-ATP6NAD4	20,11	6,25	0,00	32,00	6,05	5,96	0,00	22,45	0,91	1,64	0,00	4,25
CYTB	18,38	8,76	0,00	62,18	4,78	7,86	0,00	42,74	2,14	5,58	0,00	54,48
ITS	19,81	8,59	0,00	57,98	4,87	8,26	0,00	115,96	3,75	6,78	0,00	49,08

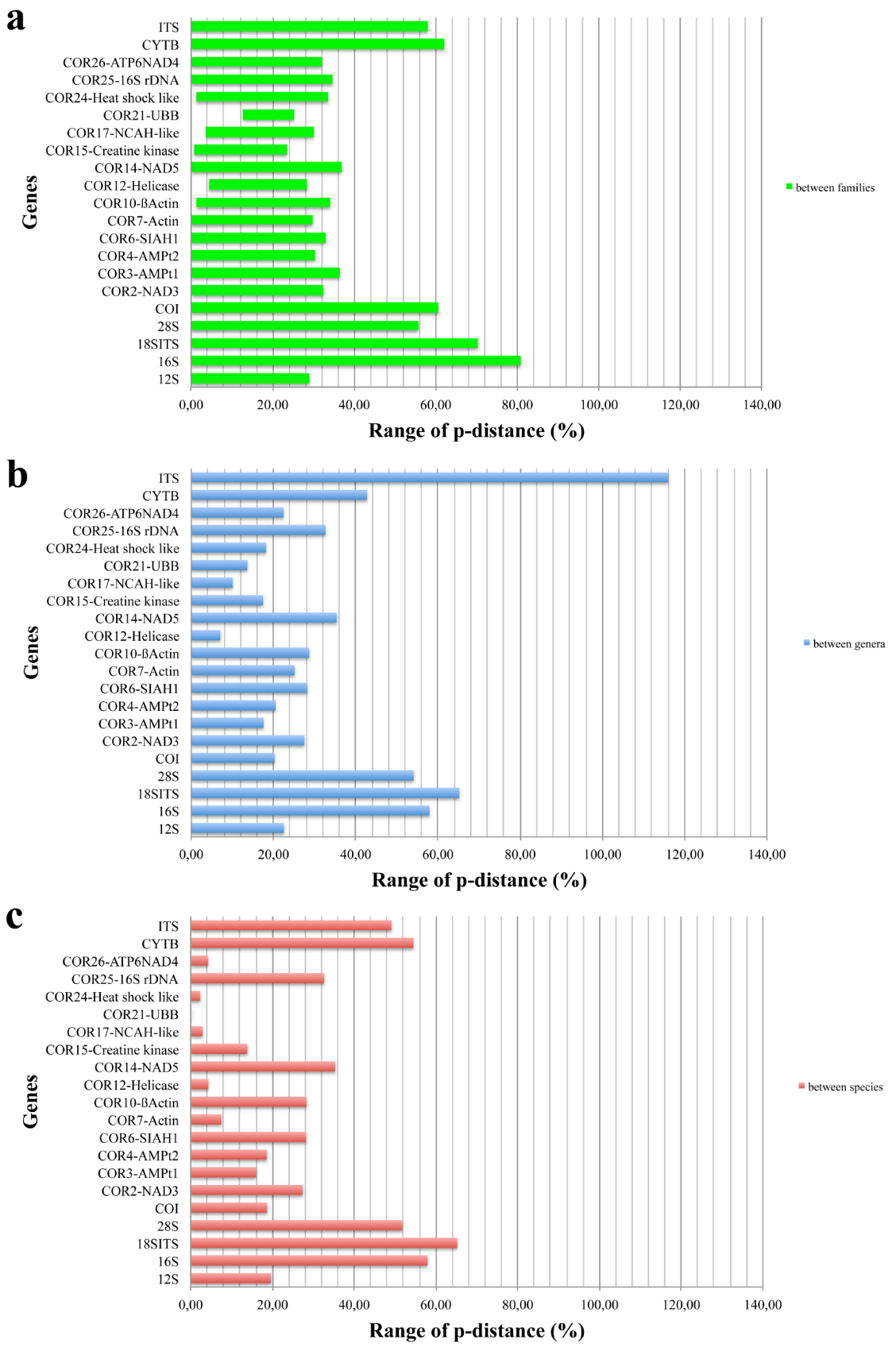

Figure 5.1a-c. Range of genetic uncorrected p-distance between scleractinian families (a), genera (b), species (c) calculated for each molecular marker.

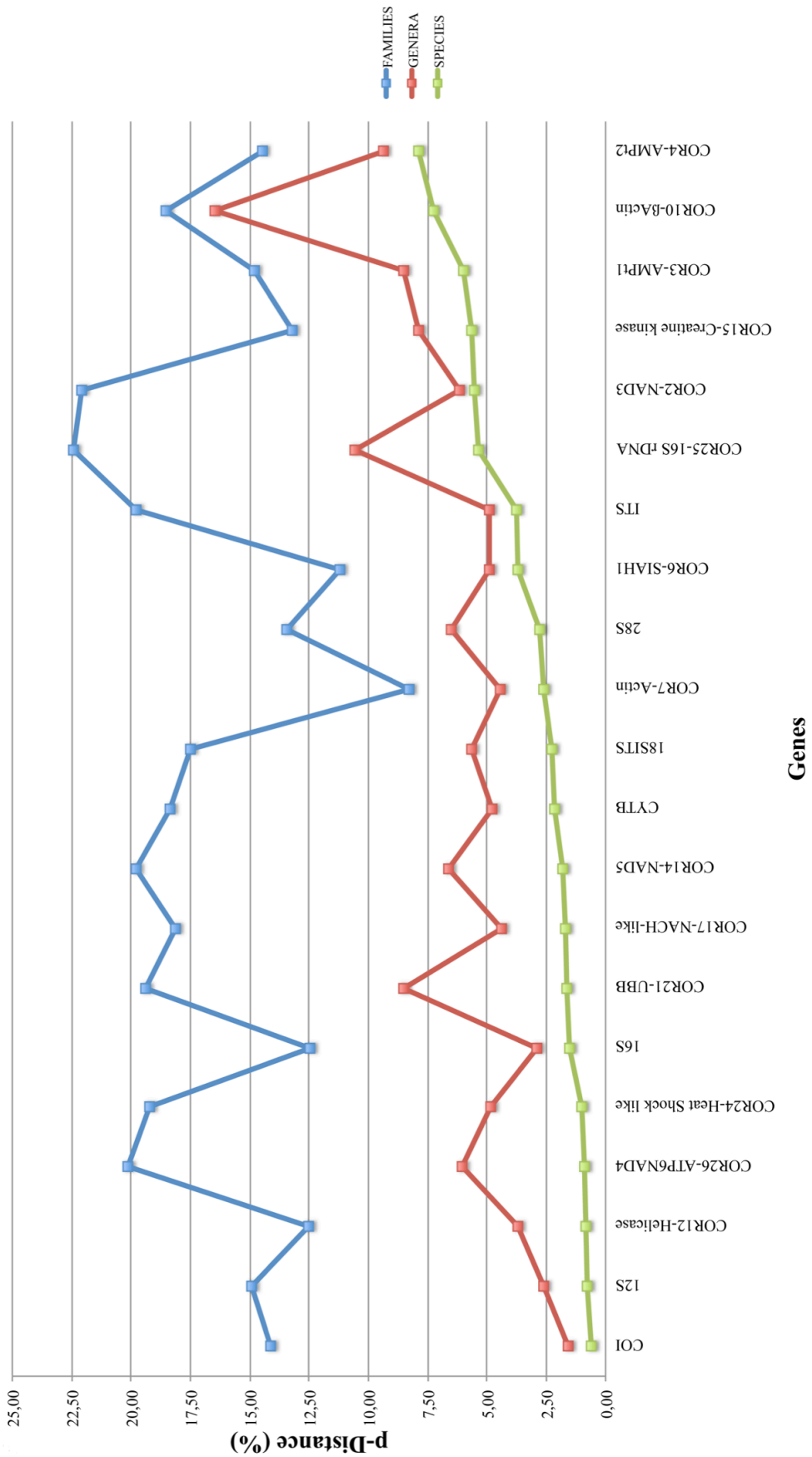

Figure 5.2. Comparison of genetic uncorrected p-distance (mean) calculated for each gene at different taxonomic level

Substitution saturation test for the old and new molecular markers showed a general pattern: transversion (Tv_3) and transition (Ti_3) mutations in the third position showed linear correlation with the growing genetic distances between pairs of taxa. The correlation attained a plateau at the boundary value of 20-30% of divergence, indicating that sequences from high distant taxa are reaching saturation levels. Exception made for markers 12S, COR4, COR7, COR12, COR17, and COR24 where saturation levels are not reached even at 35% of genetic distance. Interesting patterns were obtained for markers 16S, ITS, CYTB, 28S and COR3: the former two markers showed a "band" more than a linear correlation, where certain divergence values presented a big range of the number of Tv and Ti associated. Regarding the latter three markers, a reverse trend is observed for Tv and Ti at the boundary value of 30%, where Tv_3 outnumbered Ti_3, probably because of the transitions saturation (Fig. 5.3-5.23). Some of the markers also showed different gaps or island of values out of the range of the expected values. These cases are associated with taxonomic biases and with groups of specimens that shared only a part of the sequences length, and so, gaps simply represent absence of data with respect to another species.

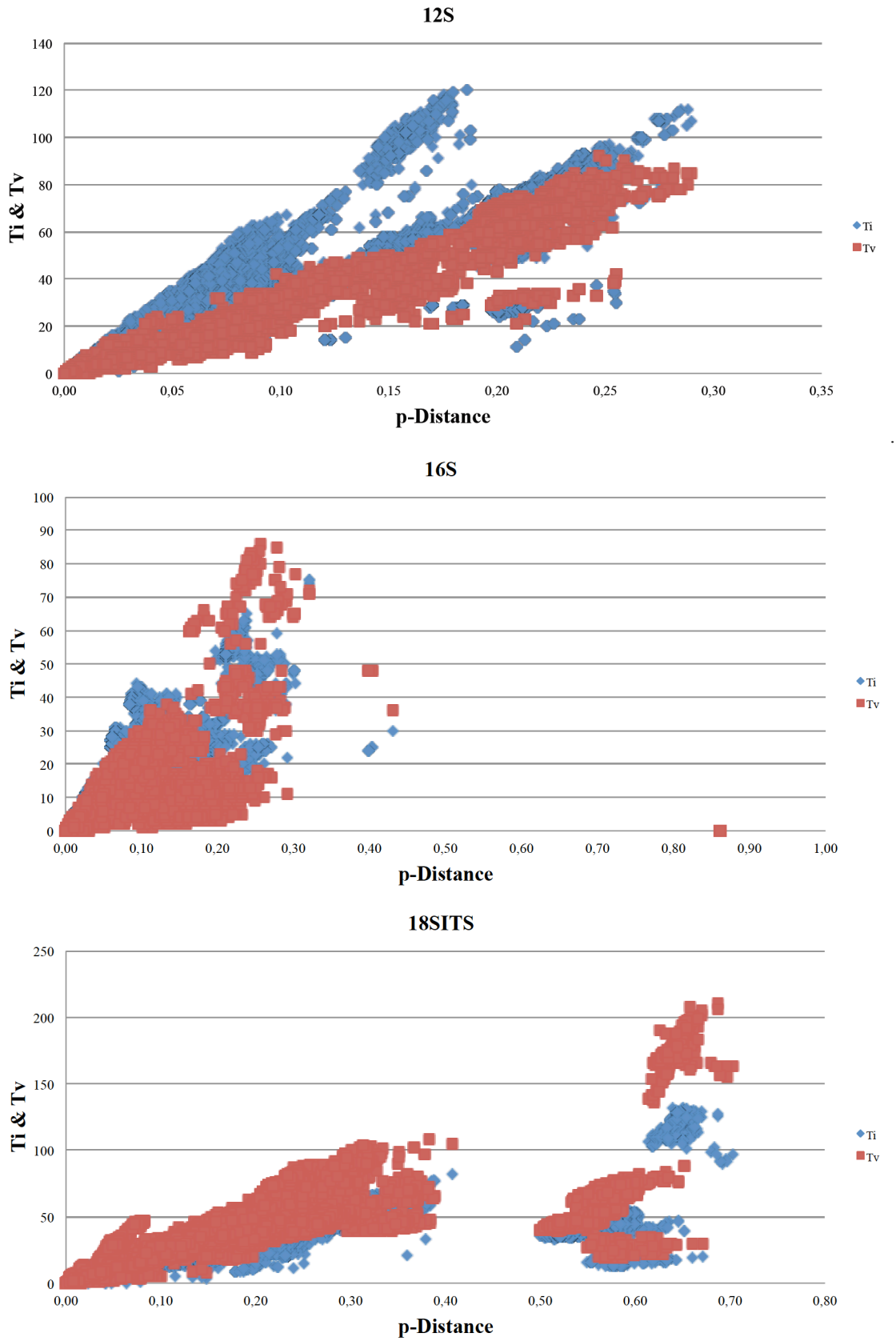

Figure 5.3-5.5. Saturation plot of transverstion (Tv) ans transition (Ti) rates against uncorrected p-distance at sum of data for 12S, 16S and 18S rDNA respectively

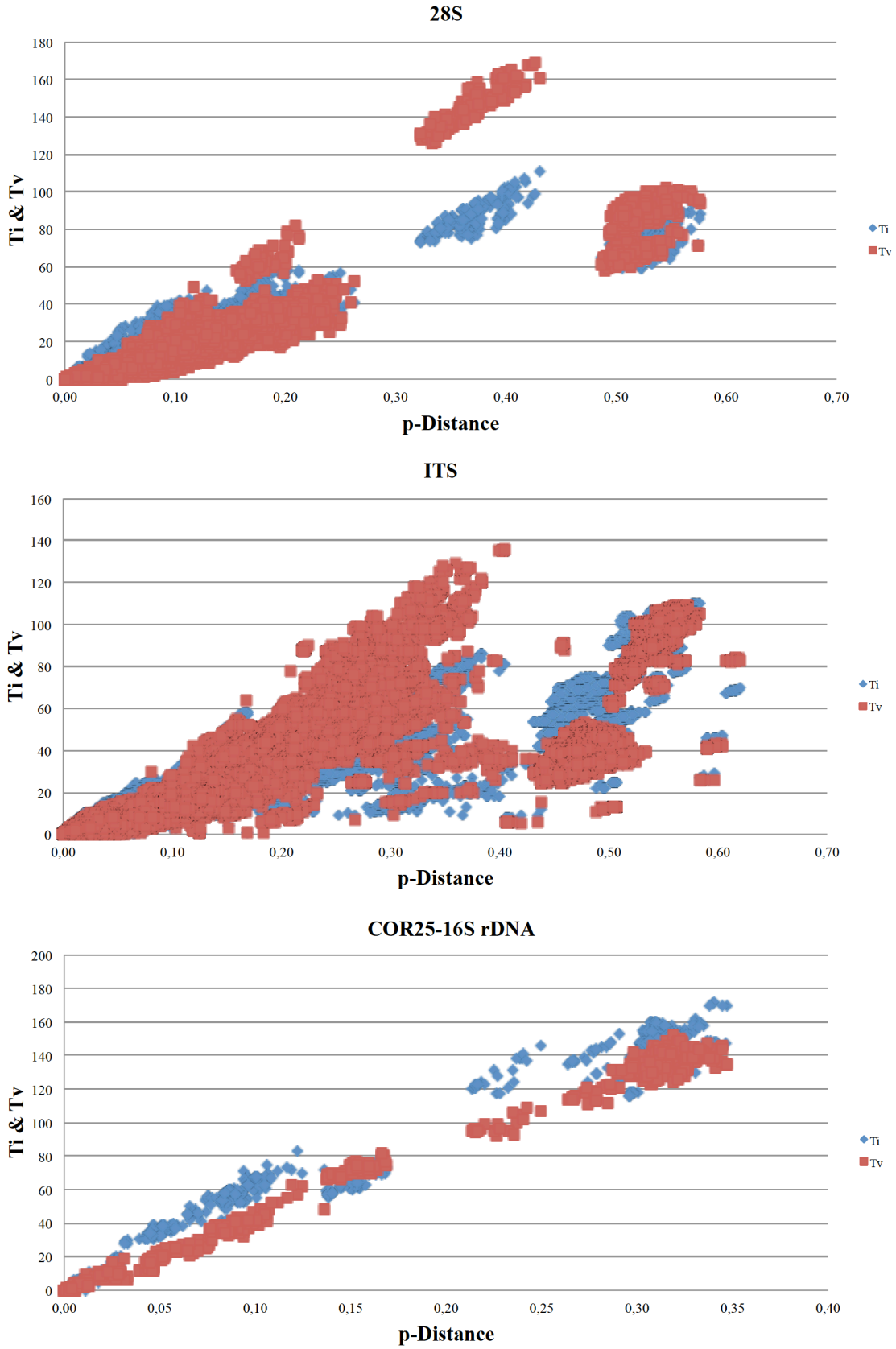

Figure 5.6-5.8. Saturation plot of transverstion (Tv) ans transition (Ti) rates against uncorrected p-distance at sum of data for 28S rDNA, ITS, and COR25-16S rDNA respectively

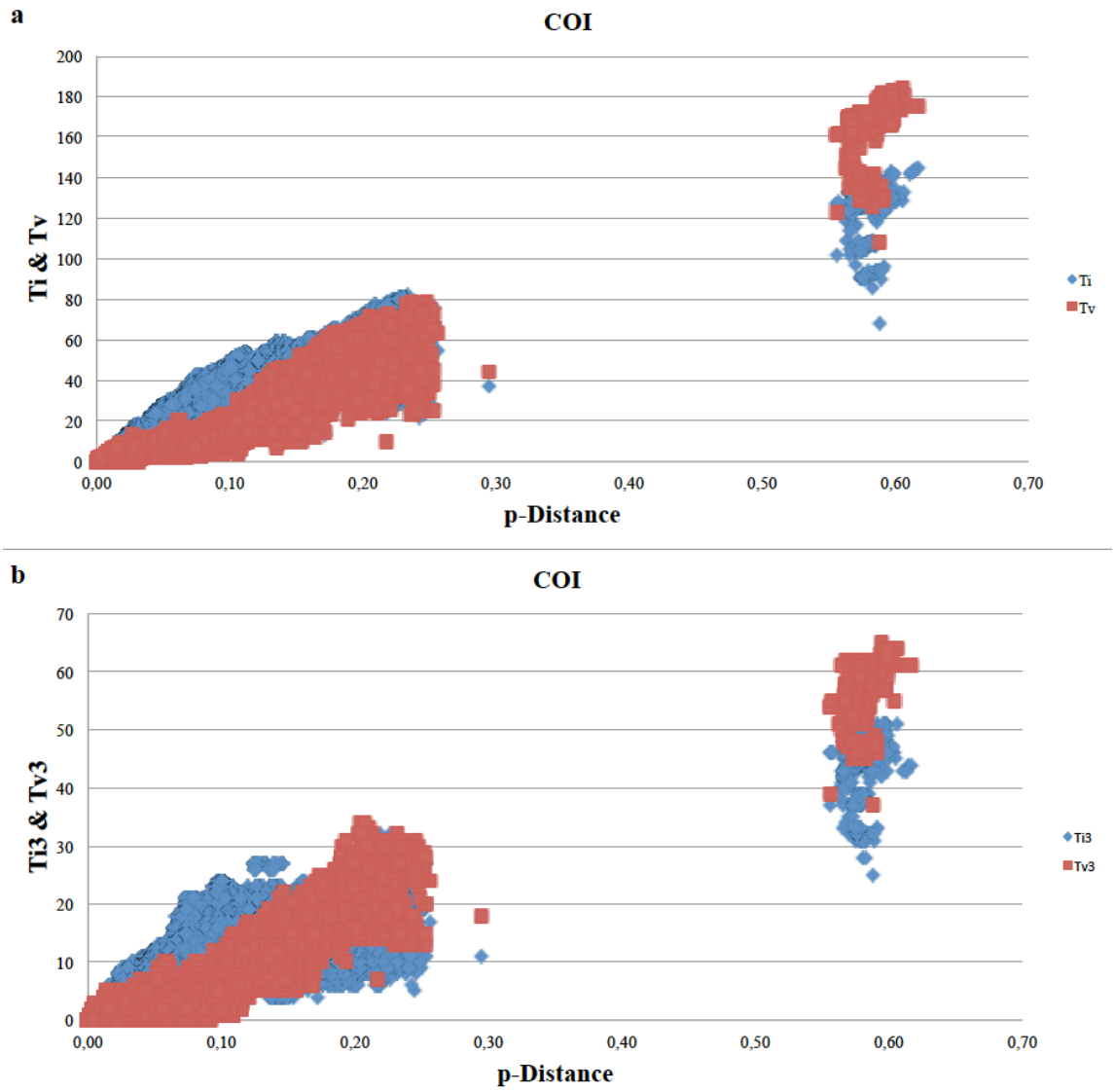

Figure 5.9. Saturation plot of transverstion (Tv) ans transition (Ti) rates against uncorrected p-distance at sum of data (a) and at third codon position (b) for COI rDNA

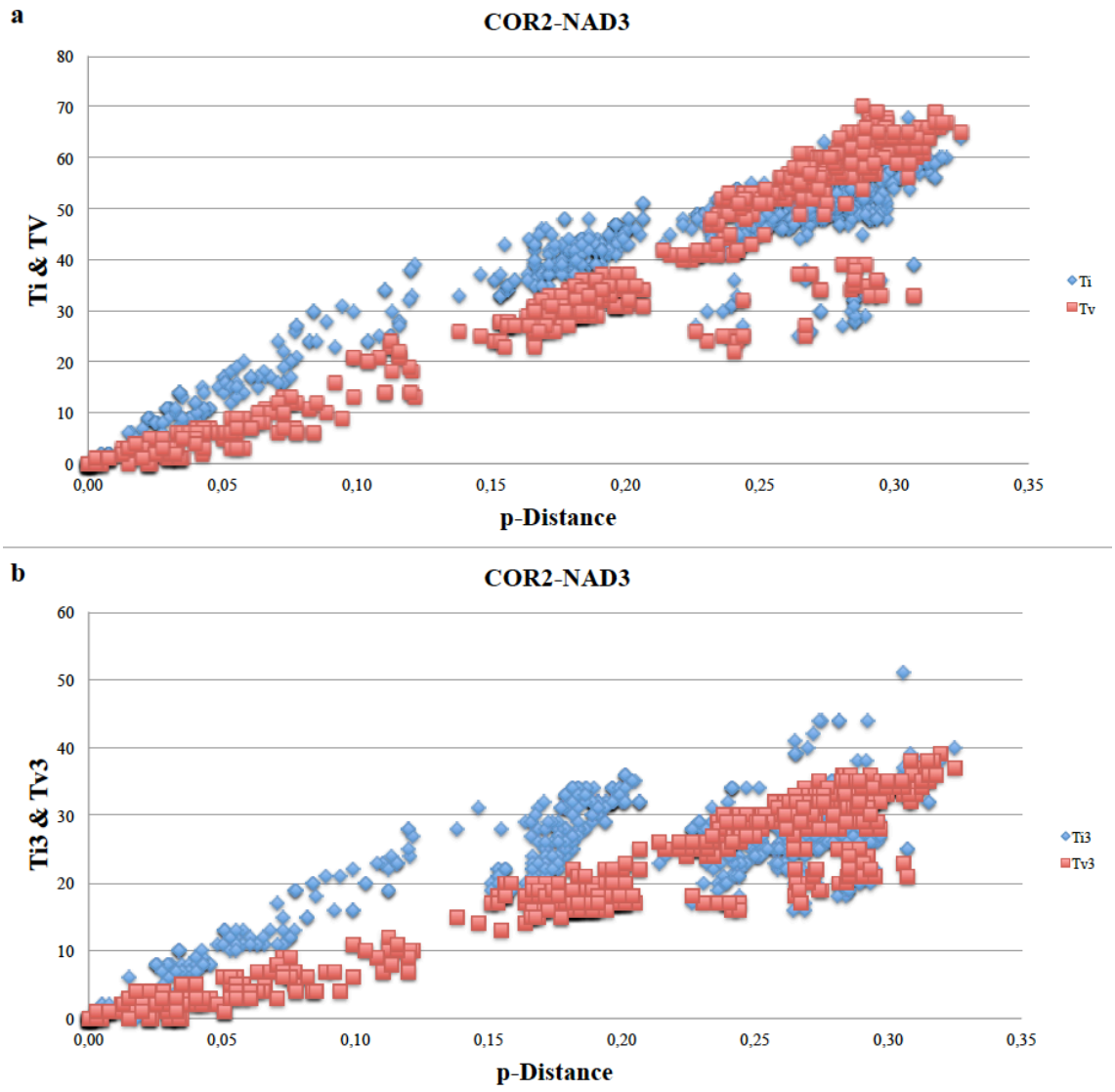

Figure 5.10. Saturation plot of transverstion (Tv) ans transition (Ti) rates against uncorrected p-distance at sum of data (a) and at third codon position (b) for COR2-NAD3

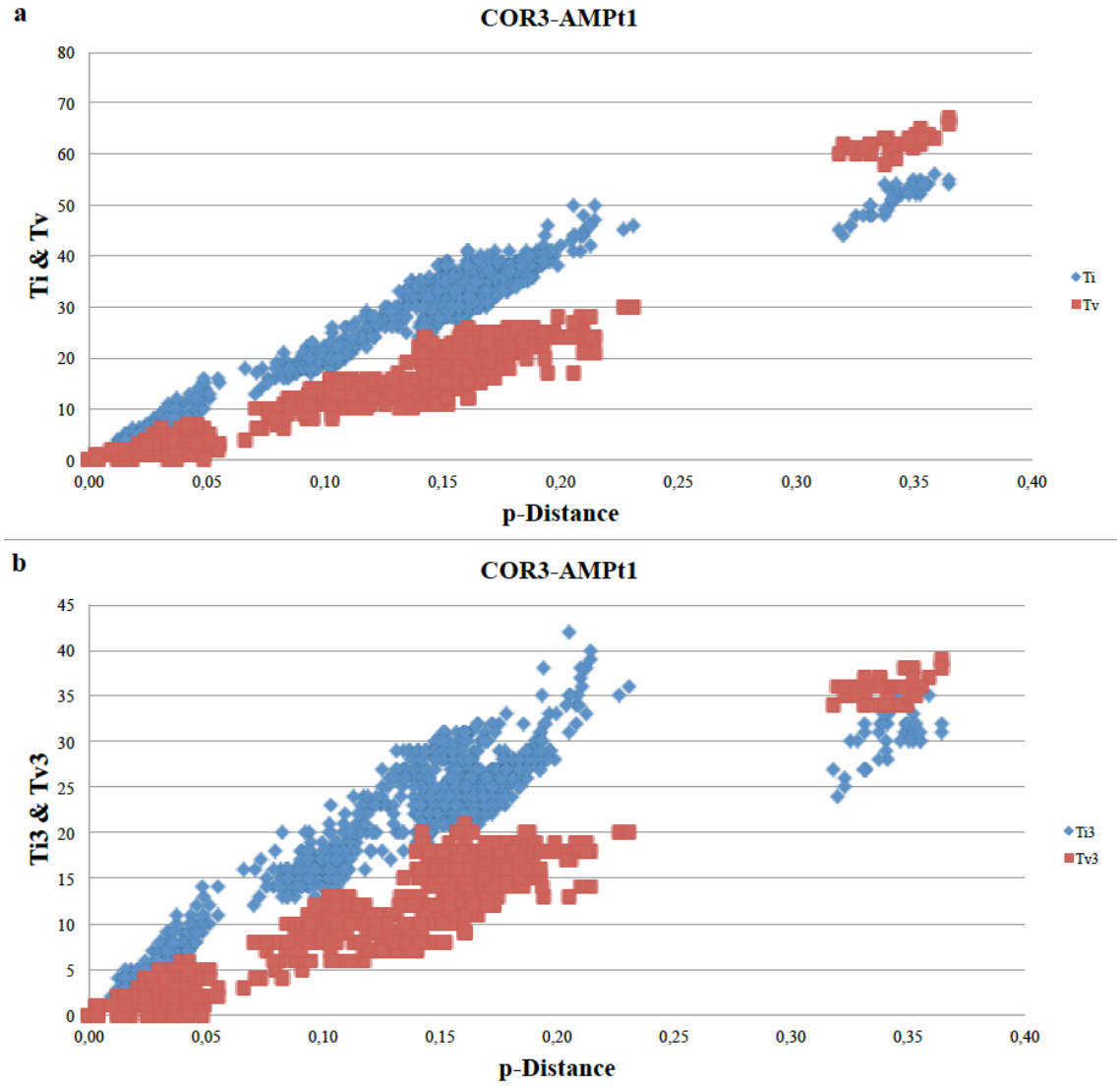

Figure 5.11. Saturation plot of transverstion (Tv) ans transition (Ti) rates against uncorrected p-distance at sum of data (a) and at third codon position (b) for COR3-AMPt1

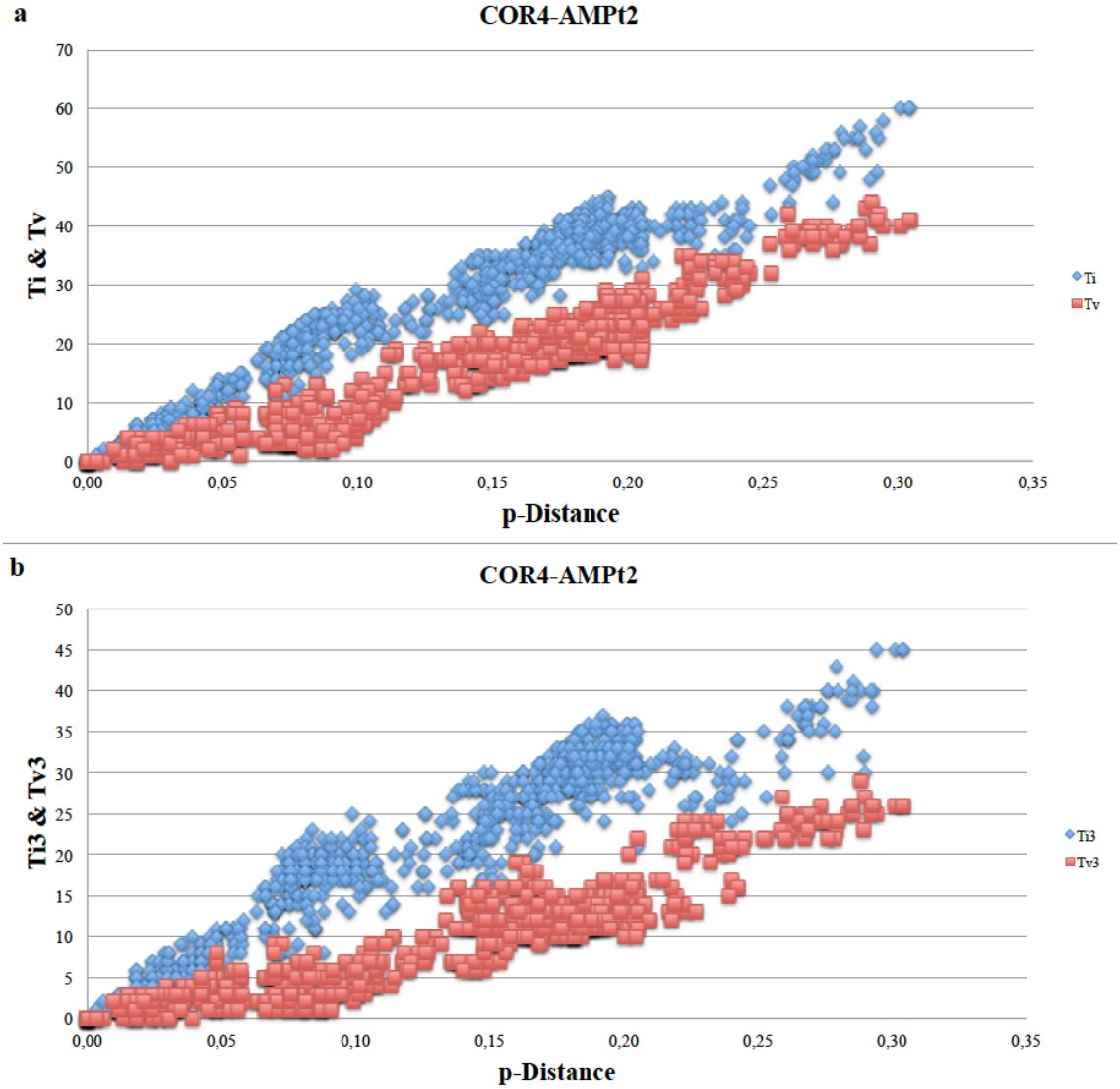

Figure 5.12. Saturation plot of transverstion (Tv) ans transition (Ti) rates against uncorrected p-distance at sum of data (a) and at third codon position (b) for COR4-AMPt2

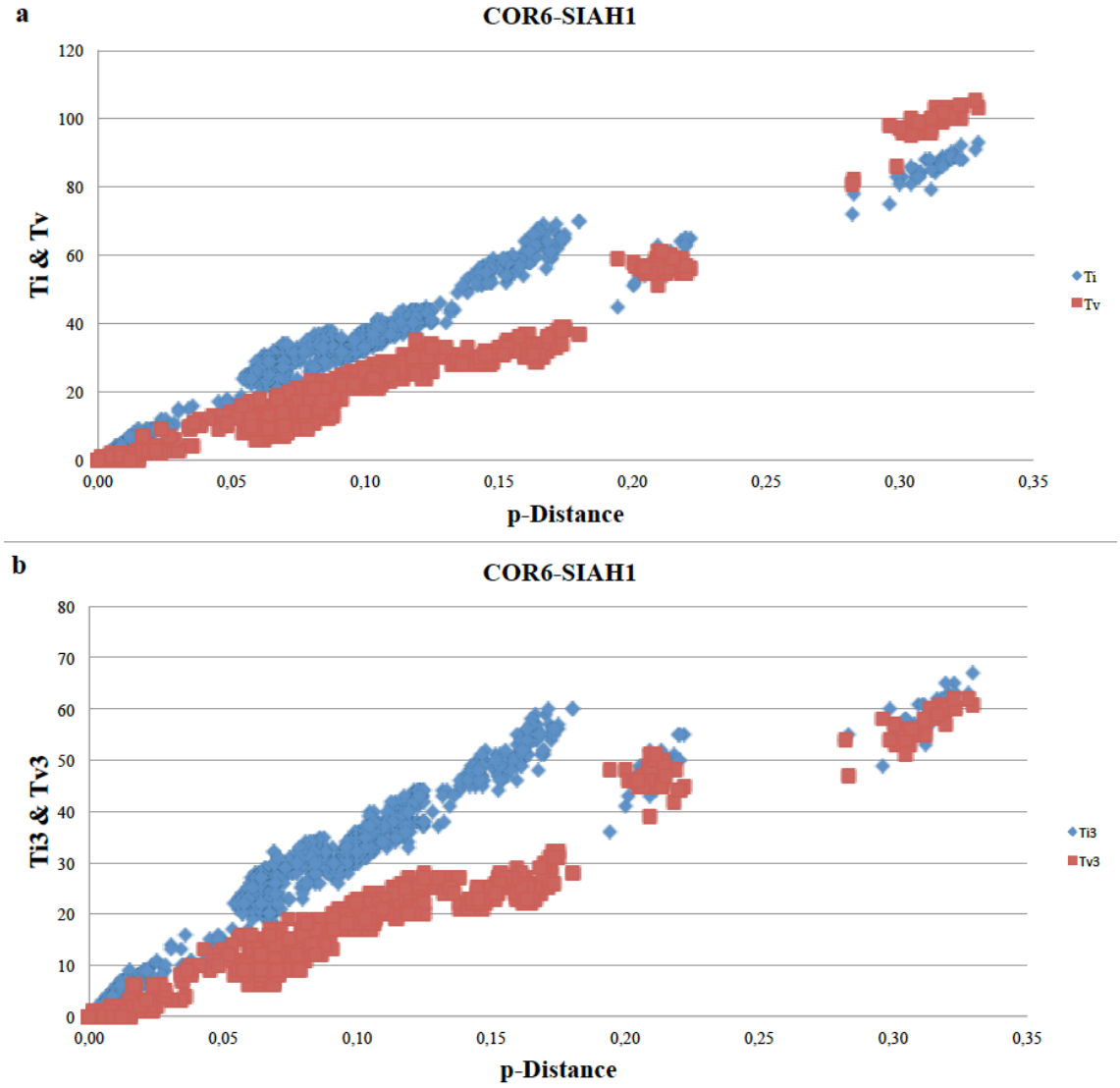

Figure 5.13. Saturation plot of transverstion (Tv) ans transition (Ti) rates against uncorrected p-distance at sum of data (a) and at third codon position (b) for COR6-SIAH1

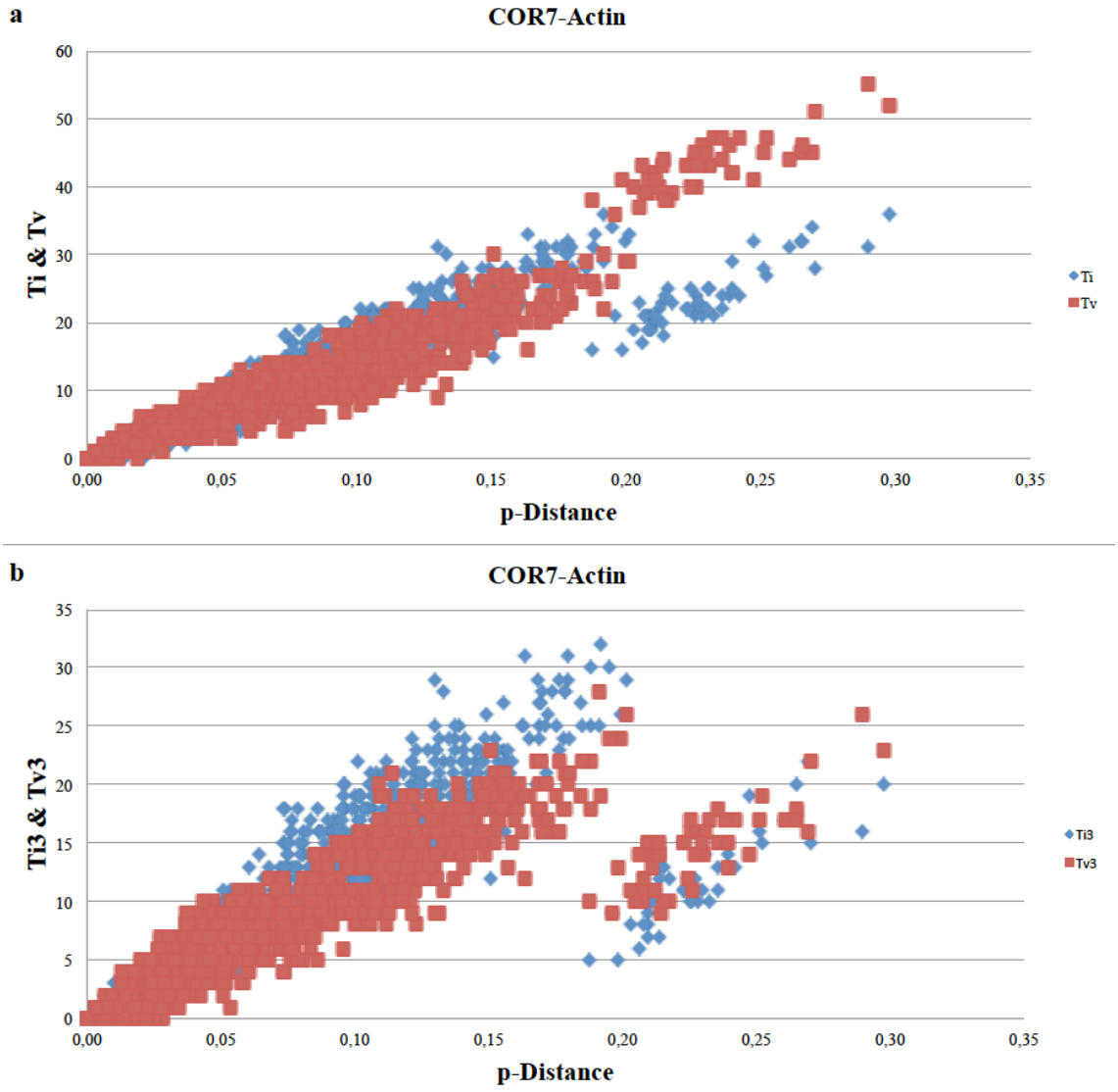

Figure 5.14. Saturation plot of transverstion (Tv) ans transition (Ti) rates against uncorrected p-distance at sum of data (a) and at third codon position (b) for COR7-Actin

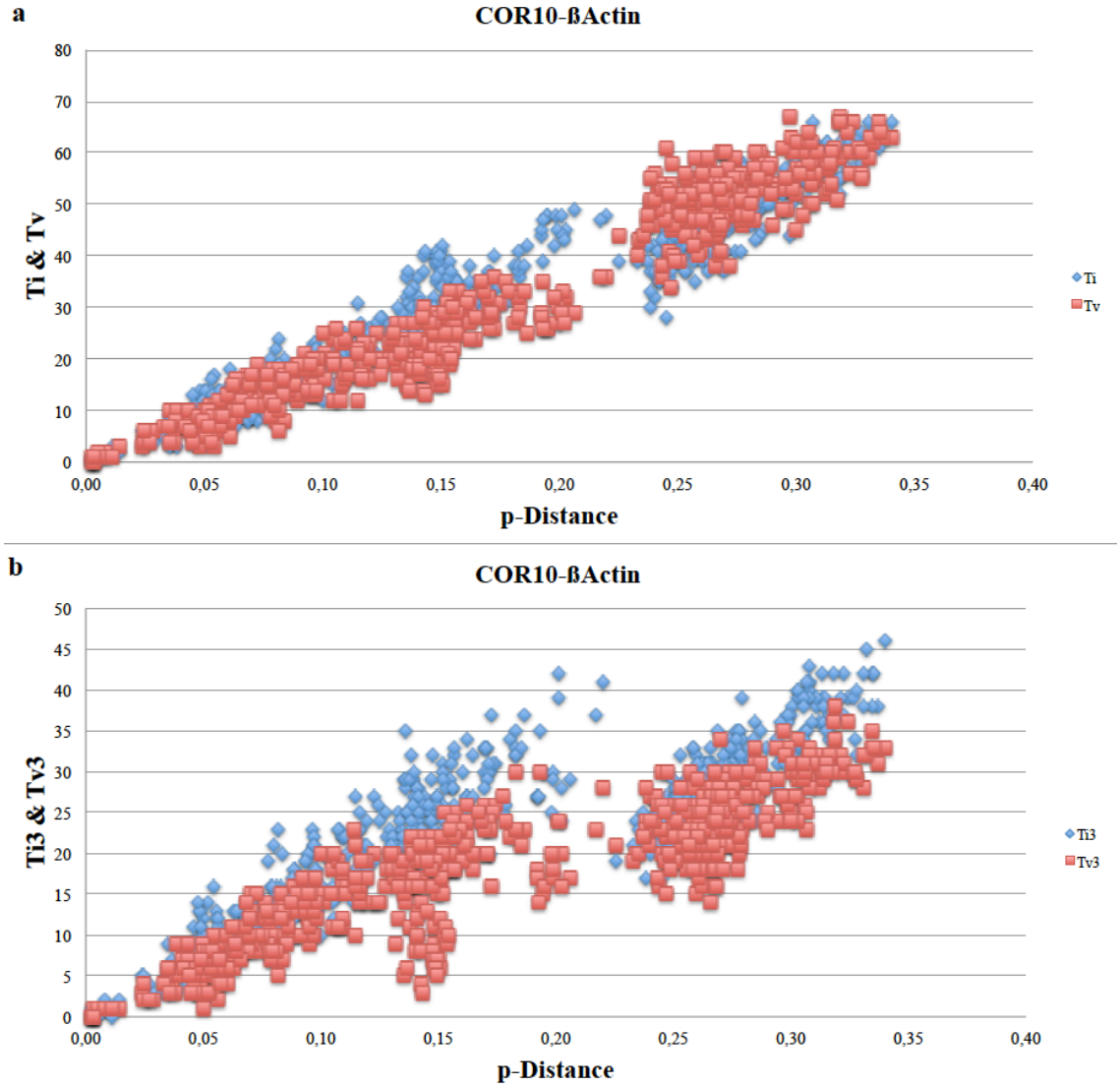

Figure 5.15. Saturation plot of transverstion (Tv) ans transition (Ti) rates against uncorrected p-distance at sum of data (a) and at third codon position (b) for COR10-ßActin

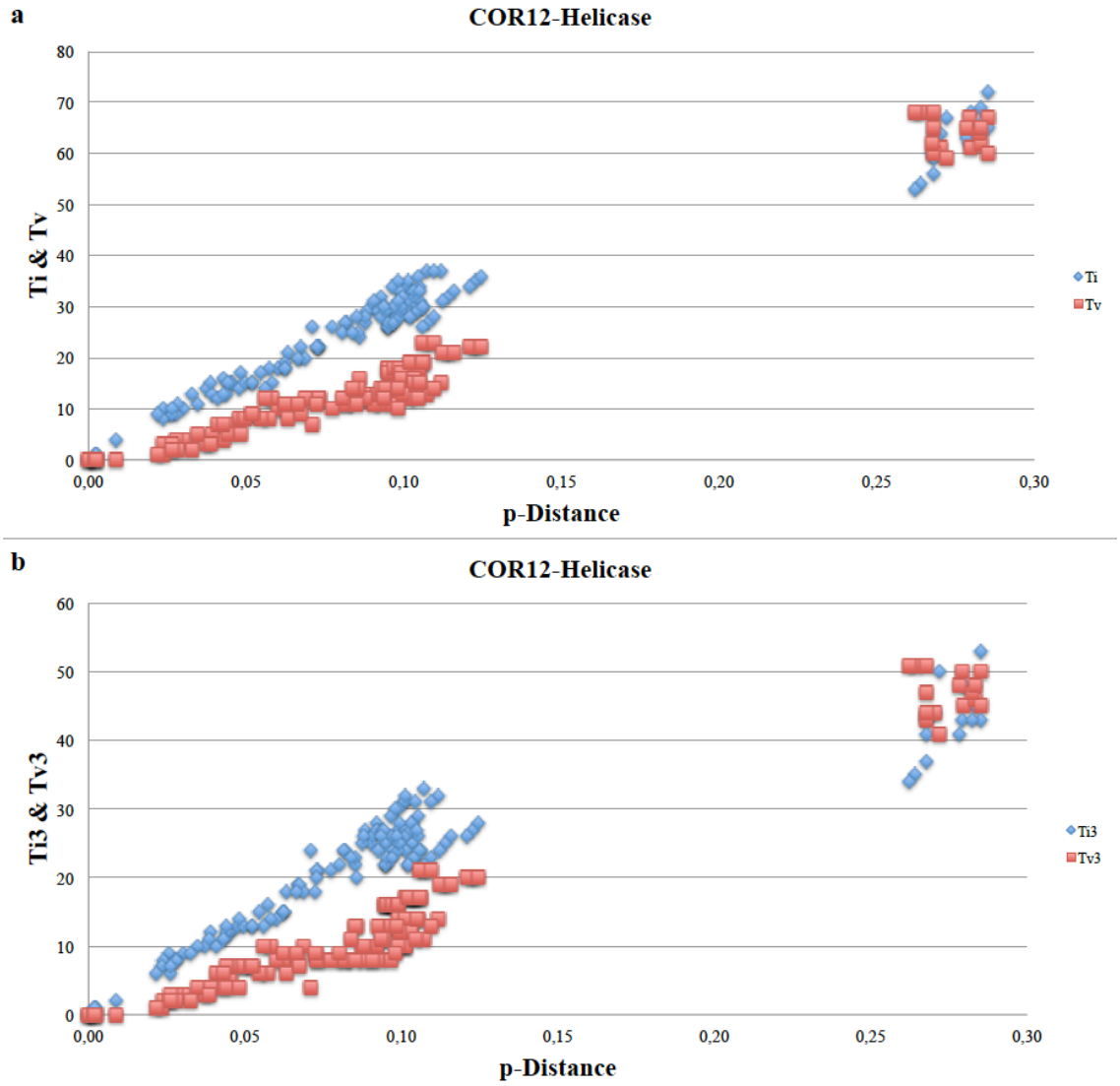

Figure 5.16. Saturation plot of transverstion (Tv) ans transition (Ti) rates against uncorrected p-distance at sum of data (a) and at third codon position (b) for COR12-Helicase

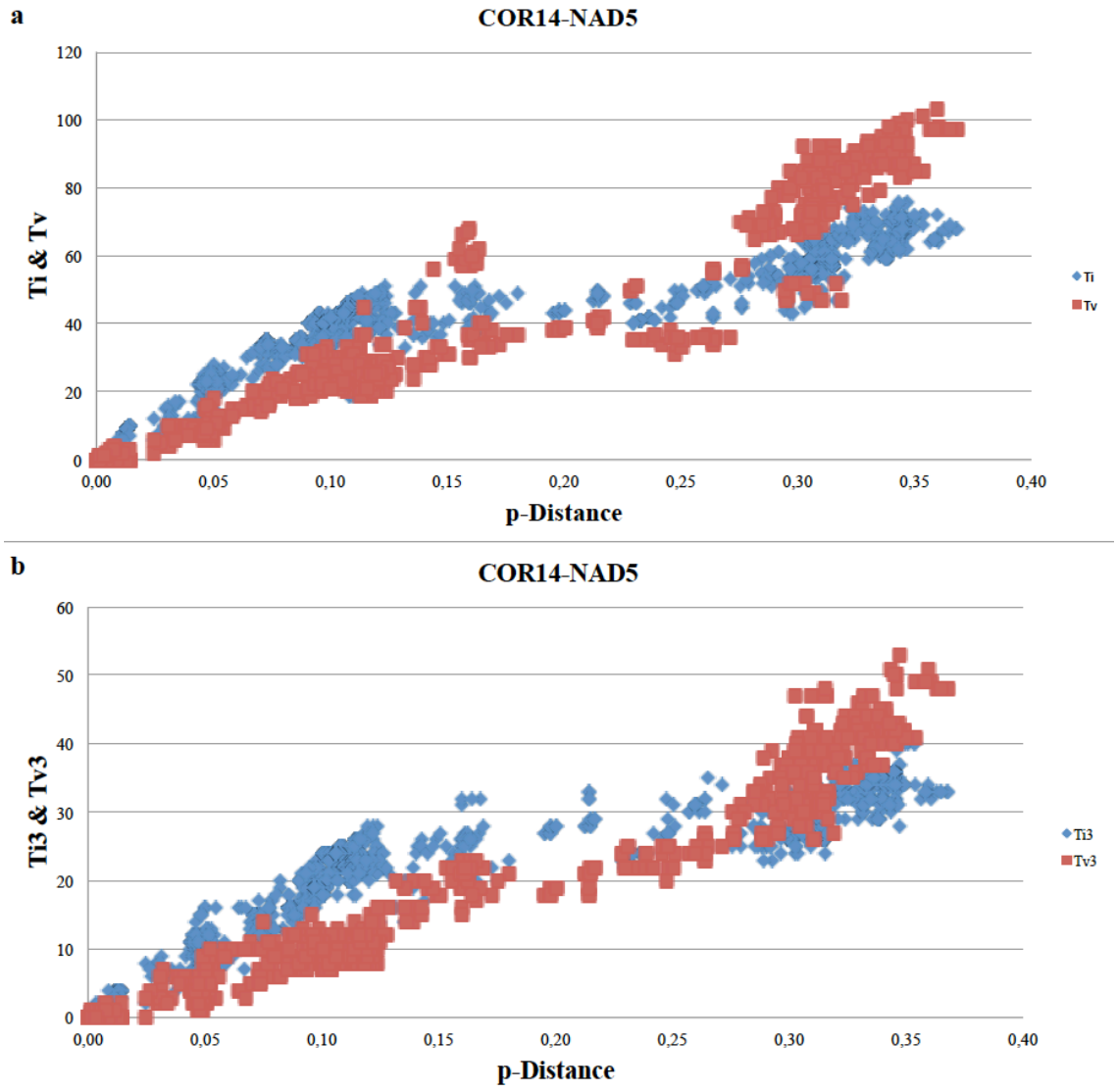

Figure 5.17. Saturation plot of transverstion (Tv) ans transition (Ti) rates against uncorrected p-distance at sum of data (a) and at third codon position (b) for COR14-NAD5

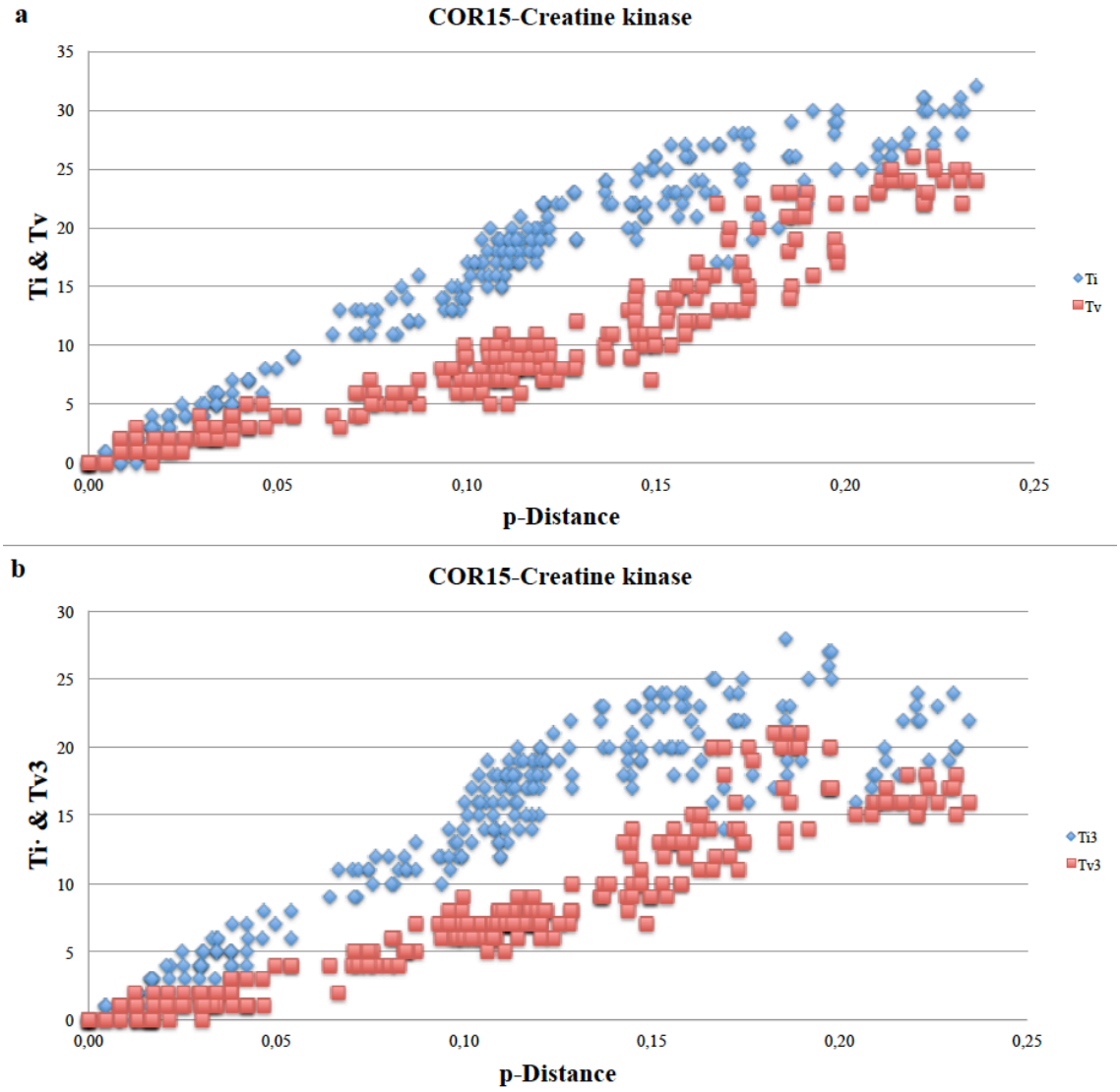

Figure 5.18. Saturation plot of transverstion (Tv) ans transition (Ti) rates against uncorrected p-distance at sum of data (a) and at third codon position (b) for COR15-Creatine kinase

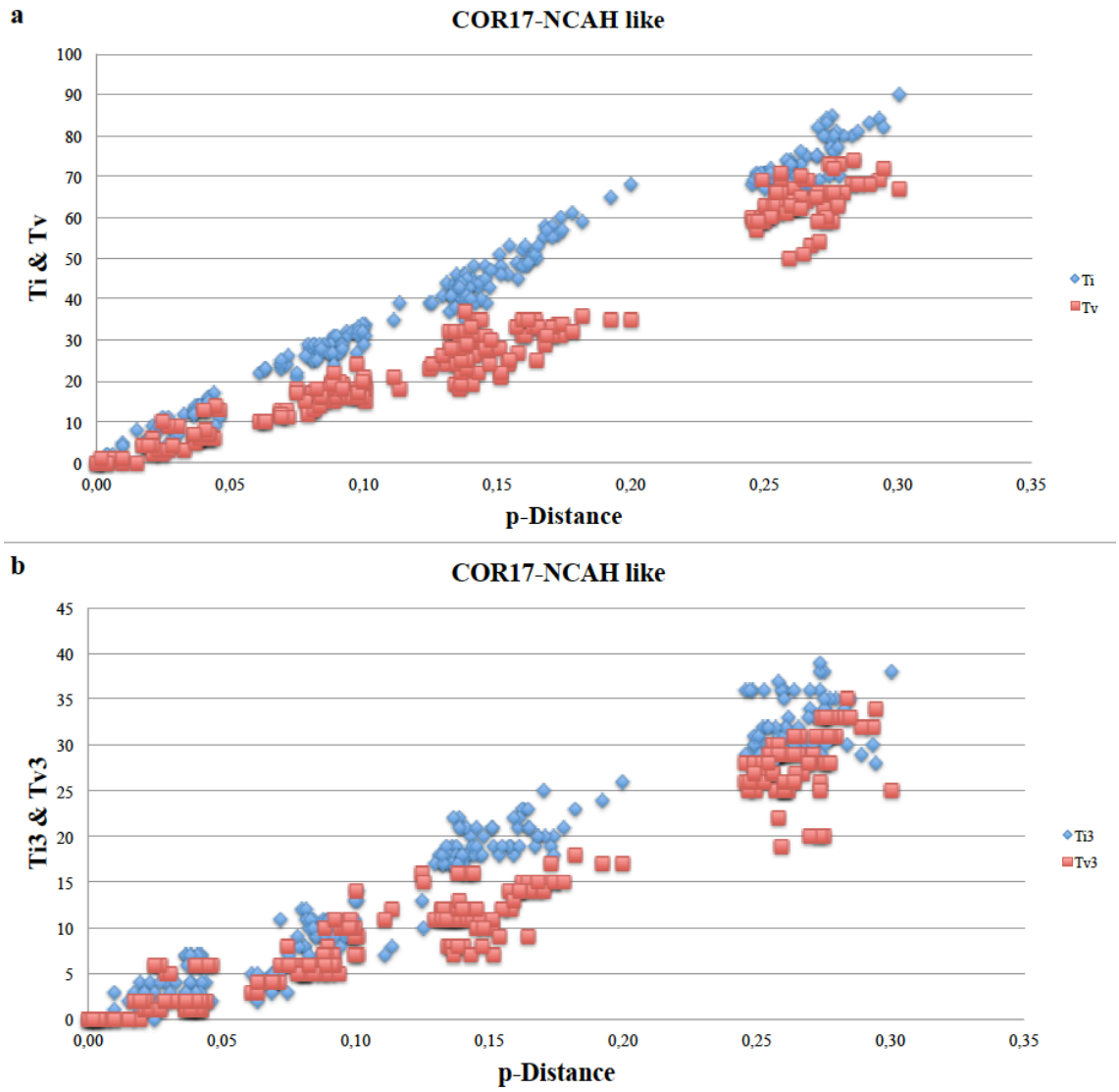

Figure 5.19. Saturation plot of transverstion (Tv) ans transition (Ti) rates against uncorrected p-distance at sum of data (a) and at third codon position (b) for COR17-NCAH like

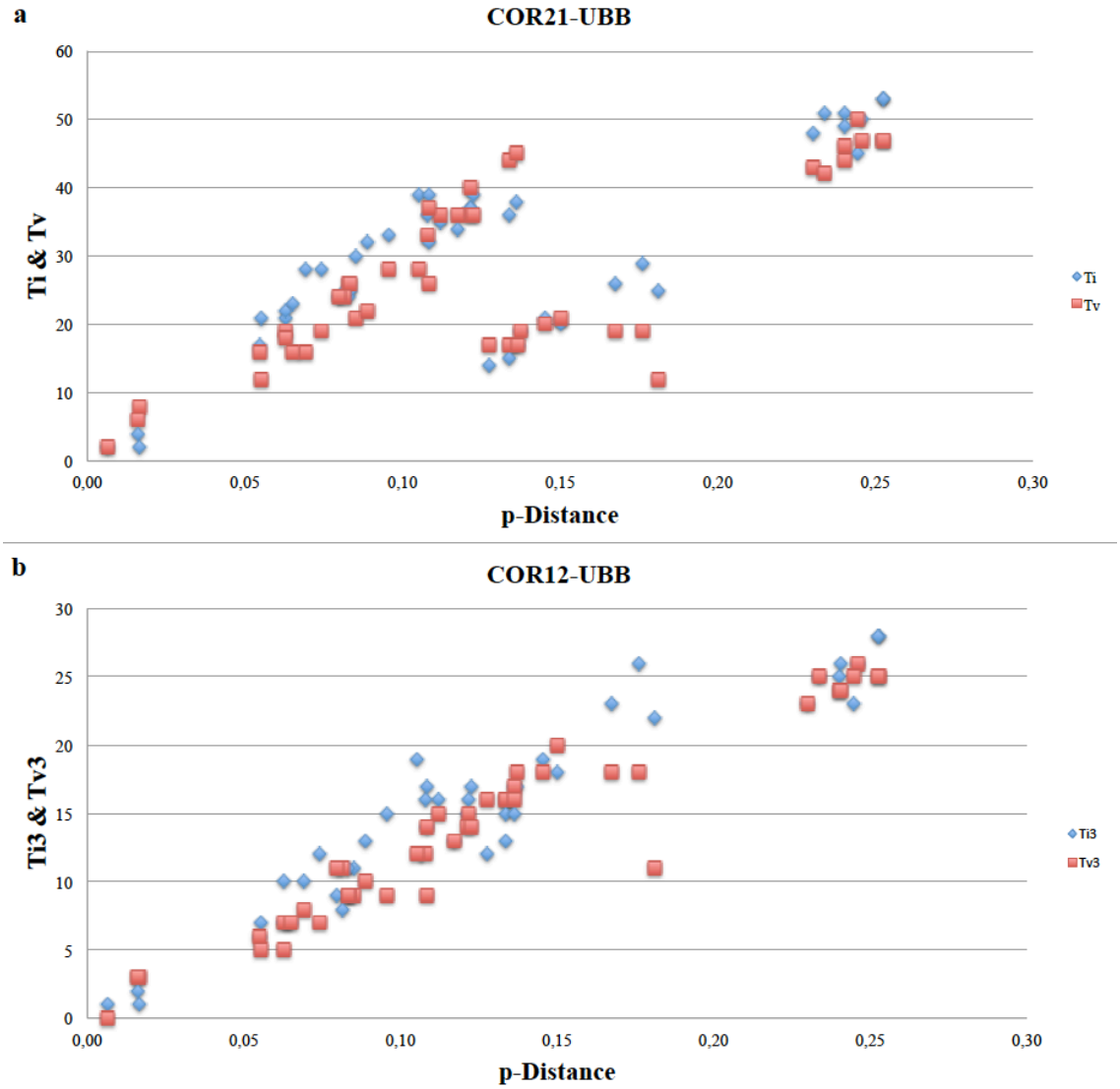

Figure 5.20. Saturation plot of transverstion (Tv) ans transition (Ti) rates against uncorrected p-distance at sum of data (a) and at third codon position (b) for COR21-UBB

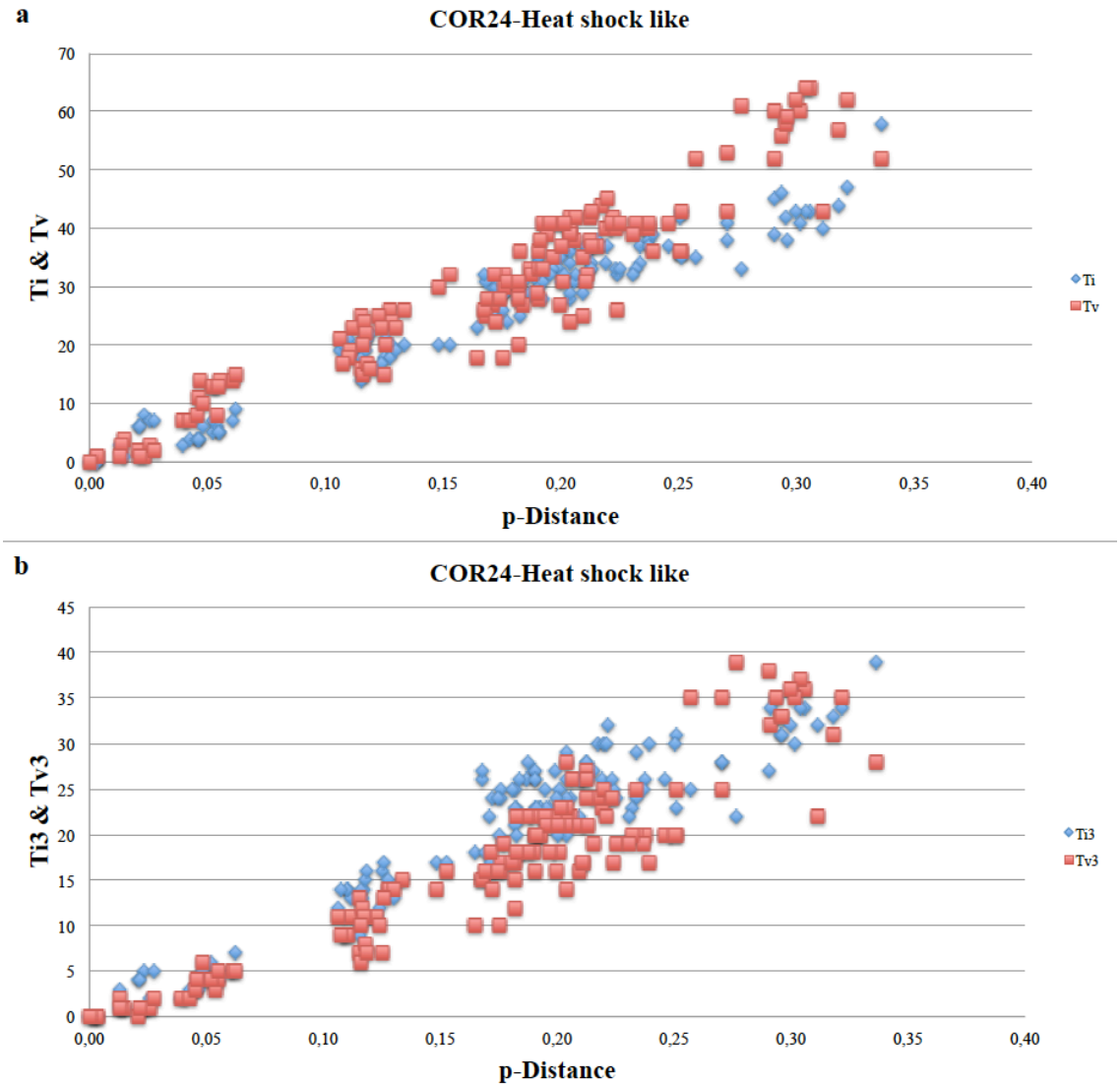

Figure 5.21. Saturation plot of transverstion (Tv) ans transition (Ti) rates against uncorrected p-distance at sum of data (a) and at third codon position (b) for COR22-Heat shock like

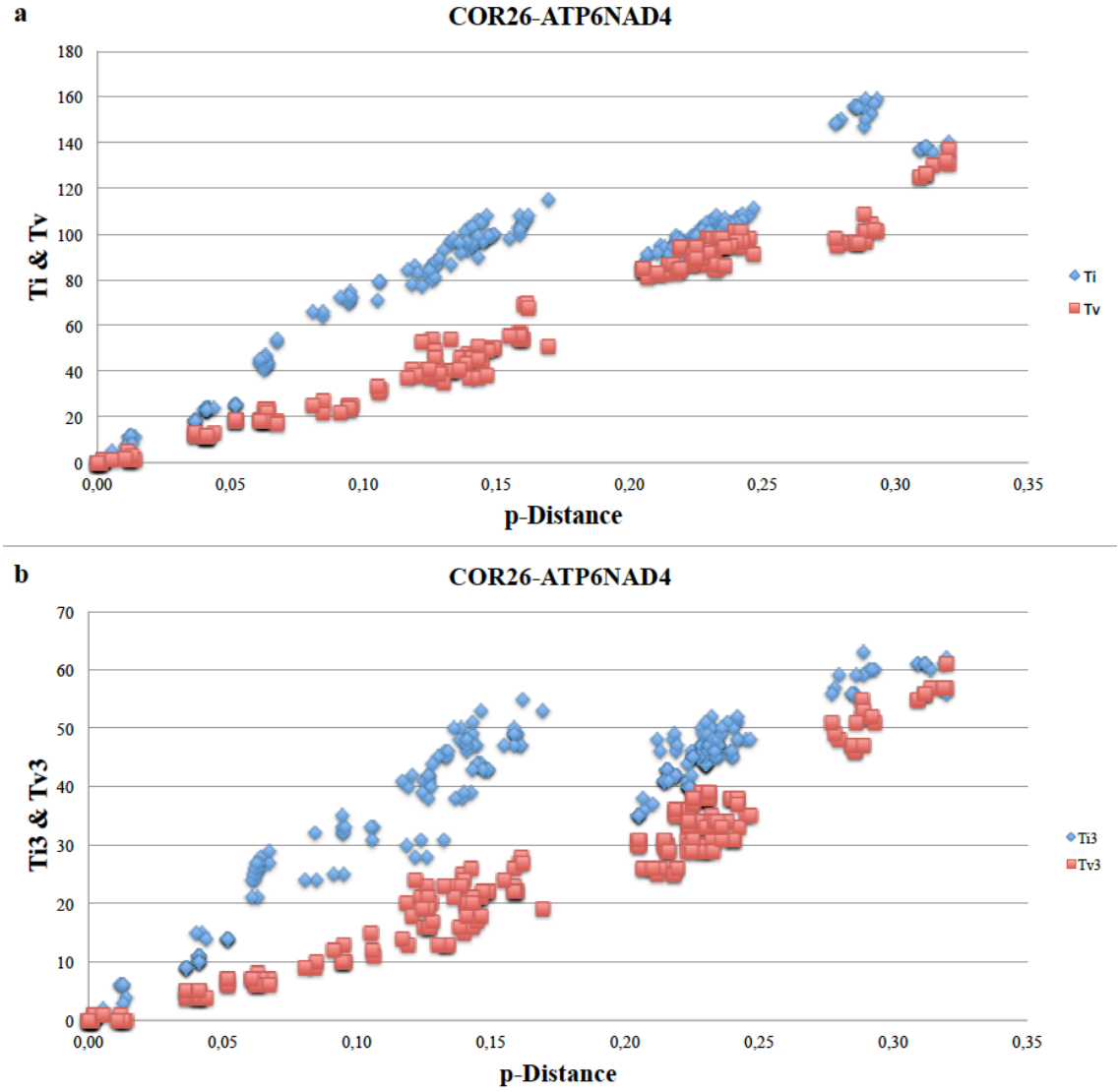

Figure 5.22. Saturation plot of transverstion (Tv) ans transition (Ti) rates against uncorrected p-distance at sum of data (a) and at third codon position (b) for COR26-ATP6NAD4

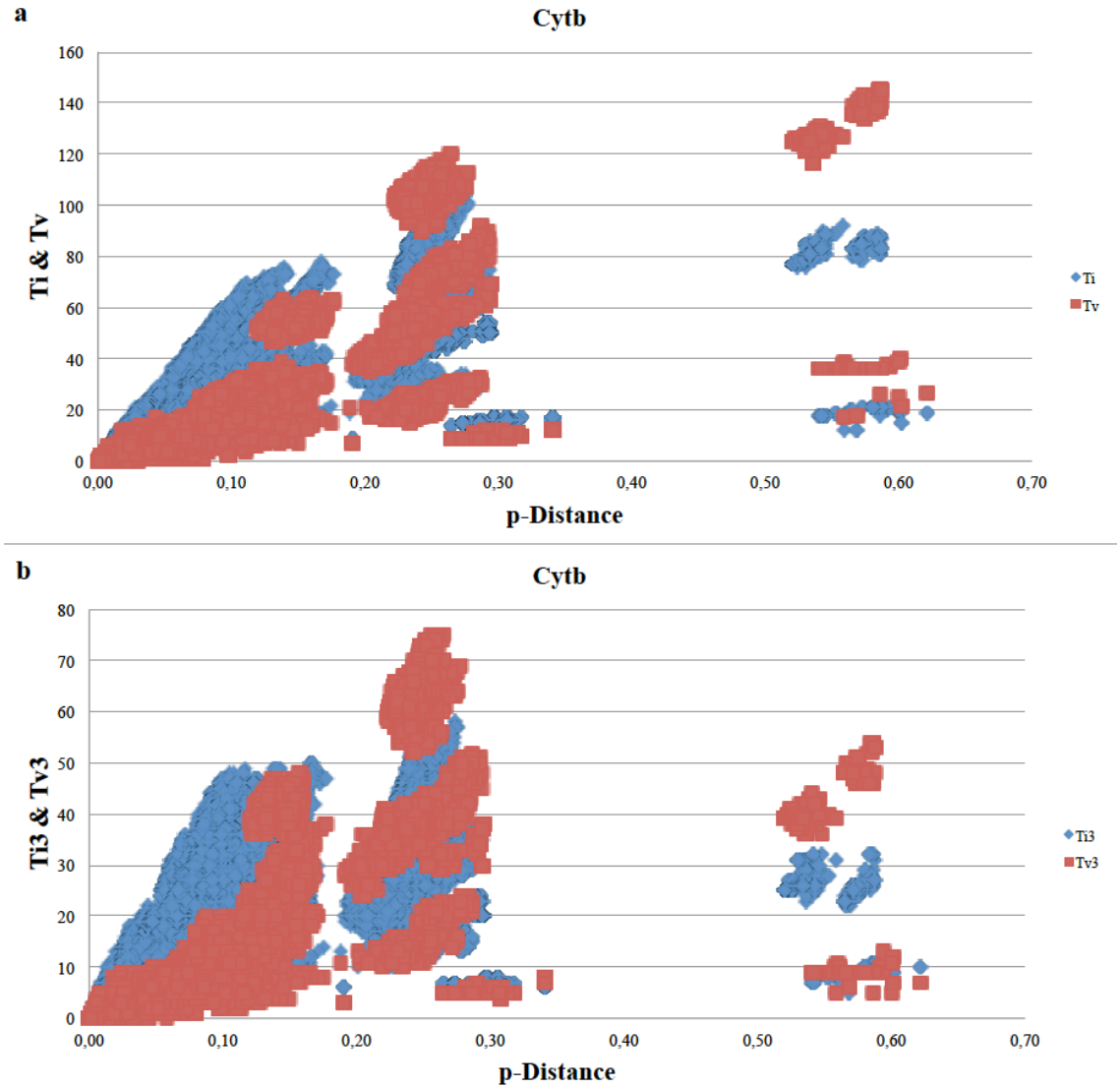

Figure 5.23. Saturation plot of transverstion (Tv) ans transition (Ti) rates against uncorrected p-distance at sum of data (a) and at third codon position (b) for Cytb

Phylogenetic MP criteria revealed a wide range of variable nucleotide parsimony-uninformative (Vpu) and parsimony-informative (Vpi) values throughout all markers: from a minimum range of 0.57% - 12.74% to a maximum range of 63.69% - 63.08% for Vpu and Vpi, respectively. The highest Vpi/Vpu ratios were found for markers COR17, CYTB, ITS, 28S, 12S, 18S and 28S (Figure 5.24, Table 5.5). COR6, 12, 15, 17 and 26 presented more than 50% of constant characters.

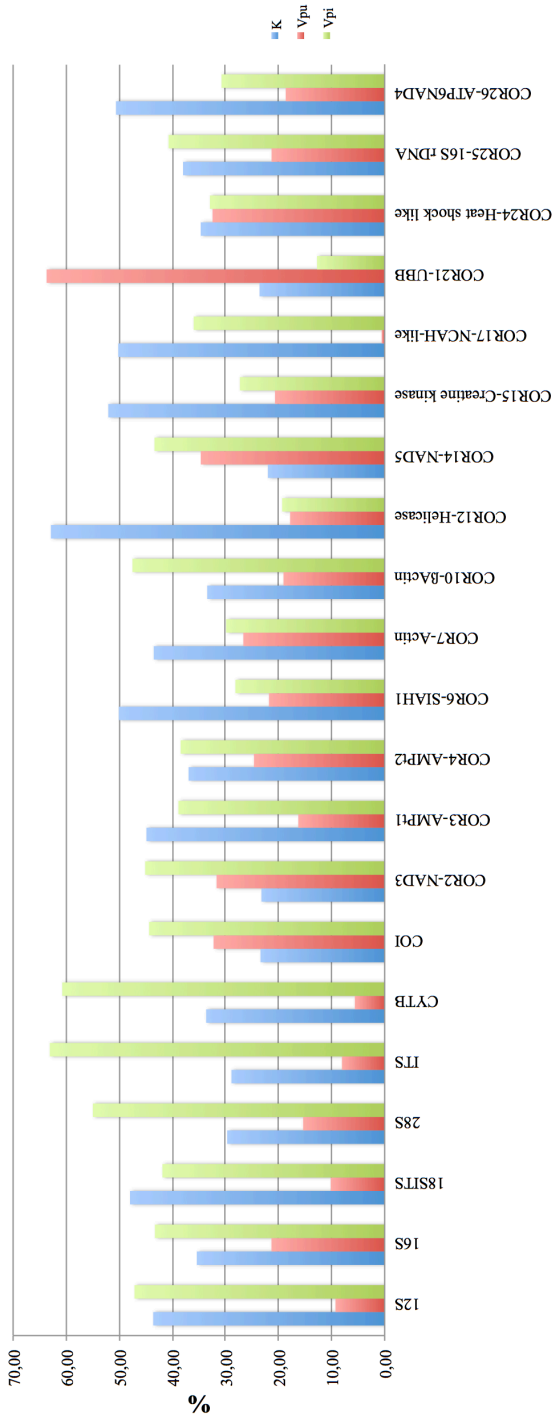

Figure 5.24. Percentage of constant nucleotide characters (K), parsimony-uninformative (Vpu) and parsimony informative (Vpi) variables throughout all the molecular markers

Table 5.5. Variable parsimony uniformative (Vpu) and informative (Vpi), and characters constant (K)

Gene	Total Nucleotides	K	Vpu	Vpi	Vpu/total	Vpi/total	Vpi/Vpu	% K
12S	993	433	92	468	9,26	47,13	5,09	43,61
16S	633	224	135	274	21,33	43,29	2,03	35,39
18SITS	1378	661	140	577	10,16	41,87	4,12	47,97
28S	1035	307	159	569	15,36	54,98	3,58	29,66
COI	667	156	215	296	32,23	44,38	1,38	23,39
COR2-NAD3	461	107	146	208	31,67	45,12	1,42	23,21
COR3-AMPt1	332	149	54	129	16,27	38,86	2,39	44,88
COR4-AMPt2	333	123	82	128	24,62	38,44	1,56	36,94
COR6-SIAH1	601	301	131	169	21,80	28,12	1,29	50,08
COR7-Actin	308	134	82	92	26,62	29,87	1,12	43,51
COR10-ßActin	383	128	73	182	19,06	47,52	2,49	33,42
COR12-Helicase	466	293	83	90	17,81	19,31	1,08	62,88
COR14-NAD5	791	174	274	343	34,64	43,36	1,25	22,00
COR15-Creatine kinase	242	126	50	66	20,66	27,27	1,32	52,07
COR17-NCAH-like	528	265	3	190	0,57	35,98	63,33	50,19
COR21-UBB	683	161	435	87	63,69	12,74	0,20	23,57
COR24-Heat shock like	404	140	131	133	32,43	32,92	1,02	34,65
COR25-16S rDNA	1314	499	280	535	21,31	40,72	1,91	37,98
COR26-ATP6NAD4	1223	619	228	376	18,64	30,74	1,65	50,61
CYTB	782	263	44	475	5,63	60,74	10,80	33,63
ITS	1081	312	87	682	8,05	63,09	7,84	28,86

These results are related in each case to the available data. In the same sense, the number of taxa and the resulting phylogenetic topologies varied amongst individual dataset. Nevertheless MP and IB criteria gave consistent results in the proposed phylogenetic relationships (Fig. 5.25-5.41) and common results were constantly present:

- 1) polyphyly of families Caryophylliidae, Dendrophylliidae, and Flabellidae;

- 2) groups of closely related taxa: A) *D. dianthus*, *Lophelia pertusa*, *Caryophyllia calveri*, *C. smithii*, *C. calveri*, *C. huinayensis*, *Pourtalosmilia anthophyllites*, *Stephanocyathus diadema*, and *Trochocyathus aithoseptatum* (Caryophylliidae); B) *O. patagonica* (Oculinidae), *Cladocora caespitosa* (incertae sedis), and *Astrangia* sp. (Rhizangiidae); C) *Dipsastraea matthai*, and *D. pallida* (Merulinidae); and even though they are not constantly present in all phylogenetic reconstructions, evidence of close relationship is showed in the following groups: D) *Stephanocyathus* (*Odontocyathus*) *coronotaus*, *P. antarctica*, *Vaughanella concinna*, and *Conotrochus funicolumna* (Caryophylliidae); E) *Acropora hemprichii*, *A. hyacynthus*, and *A. valida* (Acroporidae); F) *Astroides calycularis*, *Balanophyllia regia*, *Dendrophyllia johnsoni*, *D. ramea*, *Tubastraea aurea*, *T.micranthus*, *Rhizopsammia micranthus* (Dendrophylliidae).

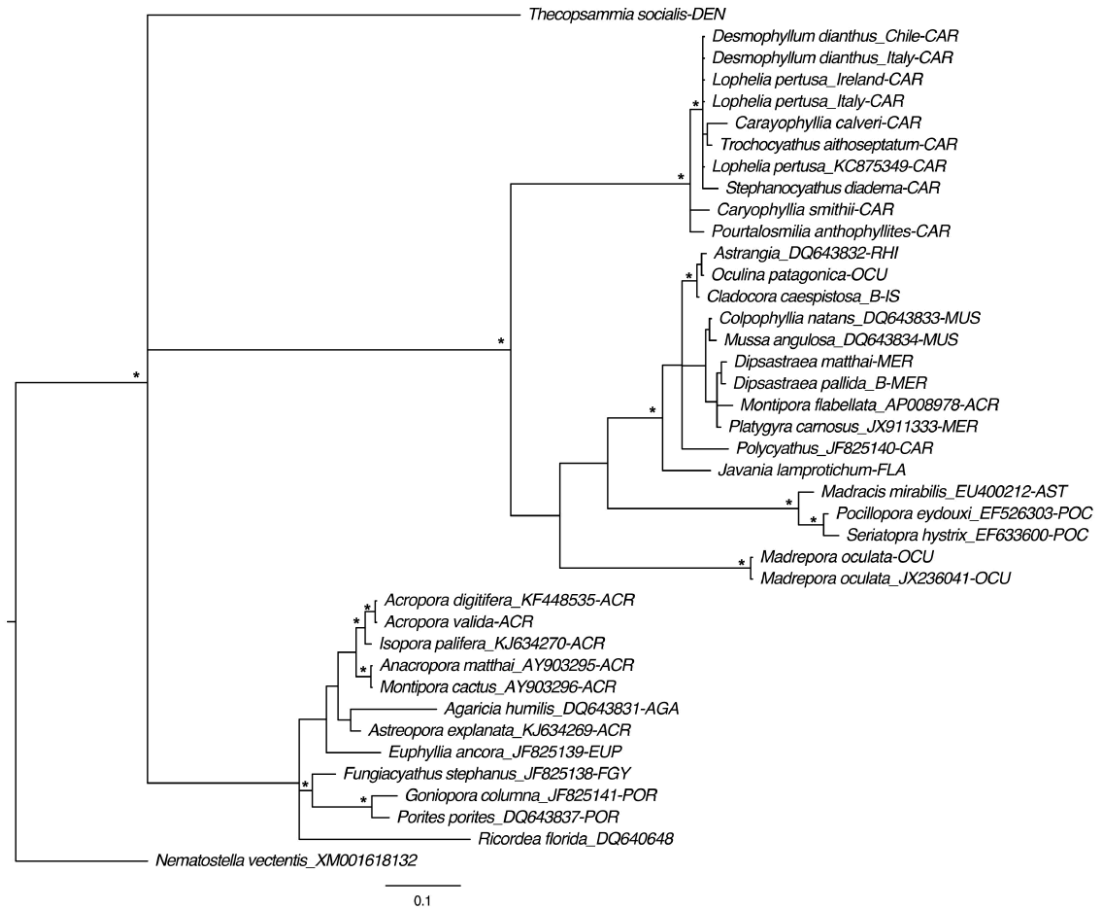

Figure 5.25. Phylogenetic reconstruction among scleractinian taxa based on COR2-NAD3. The relationship was inferred by BI and MP criteria, and asterisk (*) indicates well-supported node (pp ≥ 95; bootstrap > 70)

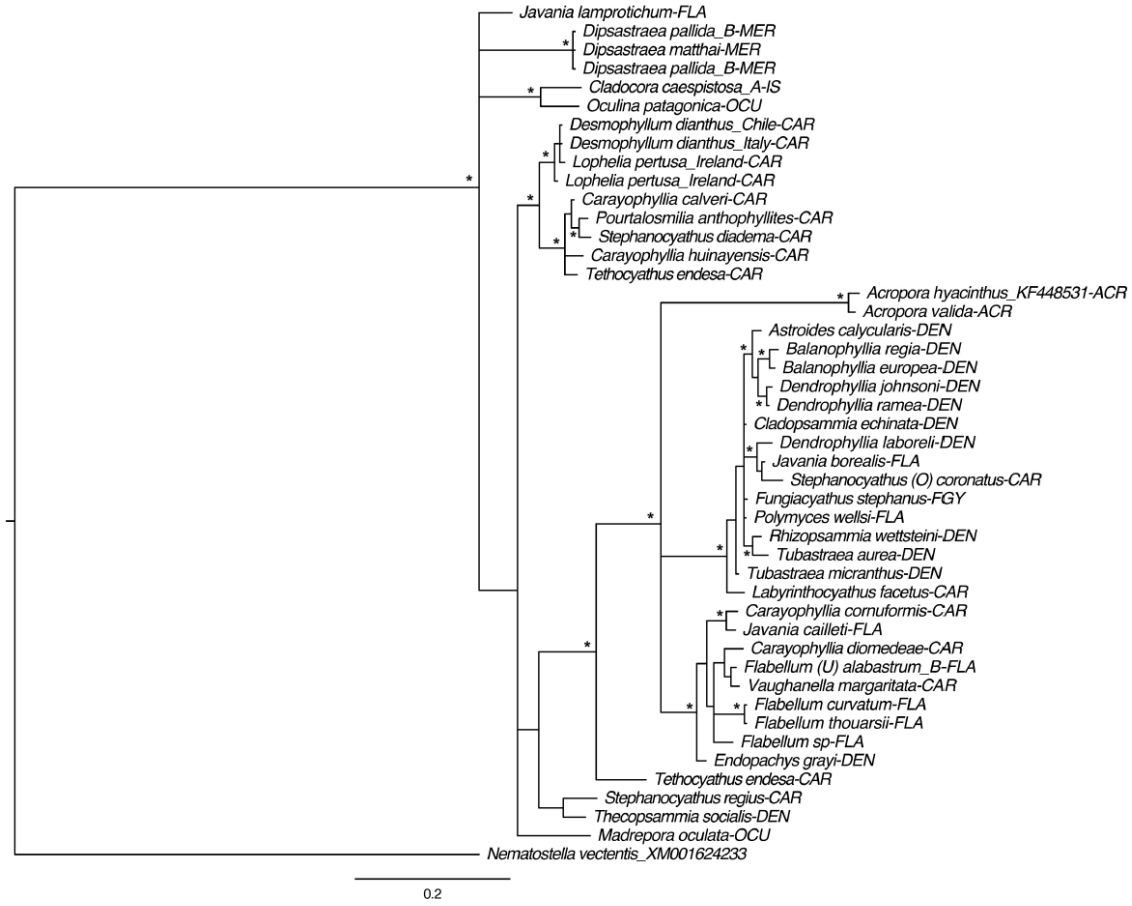

Figure 5.26. Phylogenetic reconstruction among scleractinian taxa based on COR3-AMPt1. The relationship was inferred by BI and MP criteria, and asterisk (*) indicates well-supported node (pp ≥ 95; bootstrap > 70)

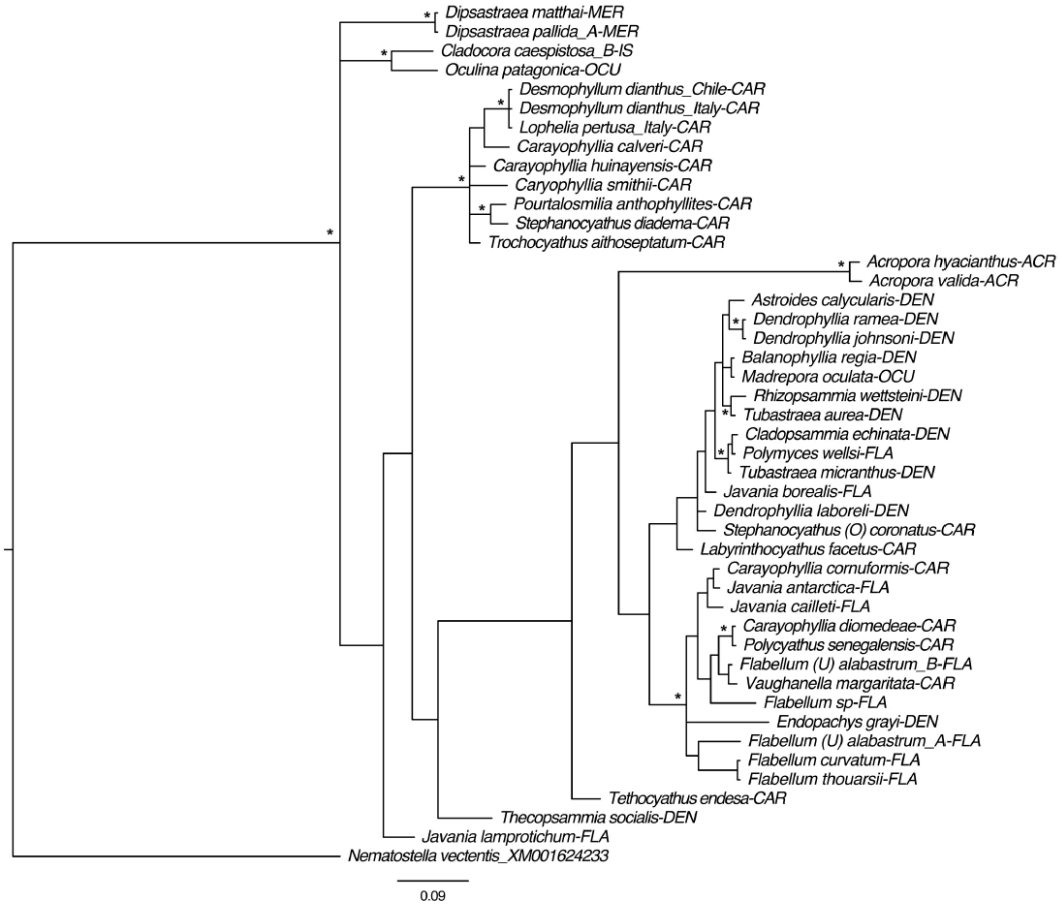

Figure 5.27. Phylogenetic reconstruction among scleractinian taxa based on COR4-AMPt2. The relationship was inferred by BI and MP criteria, and asterisk (*) indicates well-supported node (pp ≥ 95; bootstrap > 70)

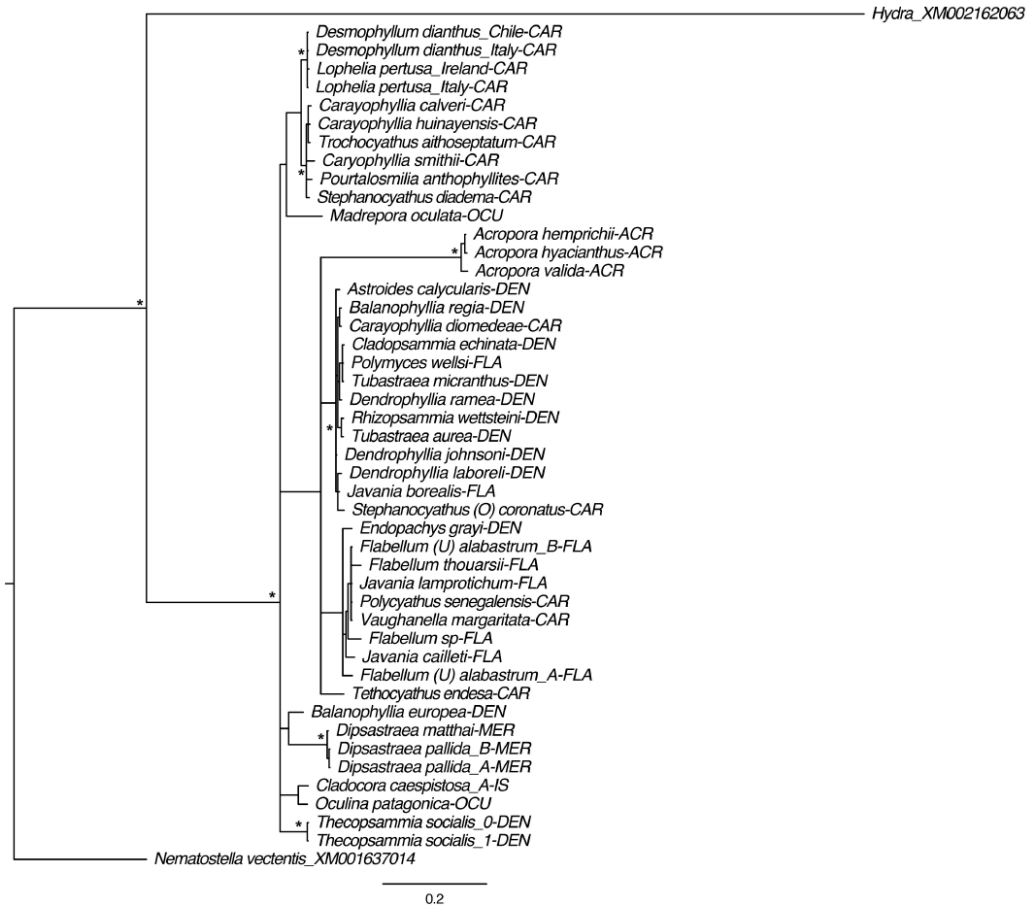

Figure 5.28. Phylogenetic reconstruction among scleractinian taxa based on COR6-SIAH1. The relationship was inferred by BI and MP criteria, and asterisk (*) indicate well-supported node (pp ≥ 95; bootstrap > 70)

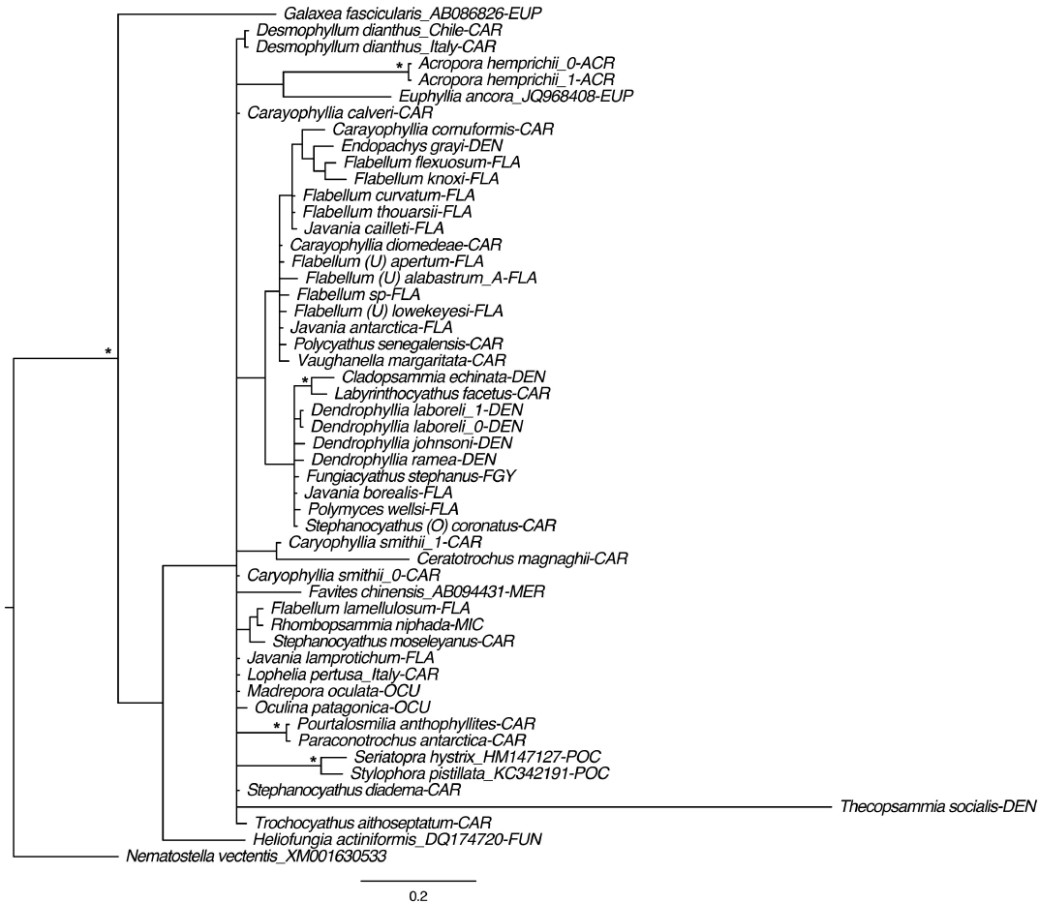

Figure 5.29. Phylogenetic reconstruction among scleractinian taxa based on COR7-Actin. The relationship was inferred by BI and MP criteria, and asterisk (*) indicates well-supported node (pp ≥ 95; bootstrap > 70)

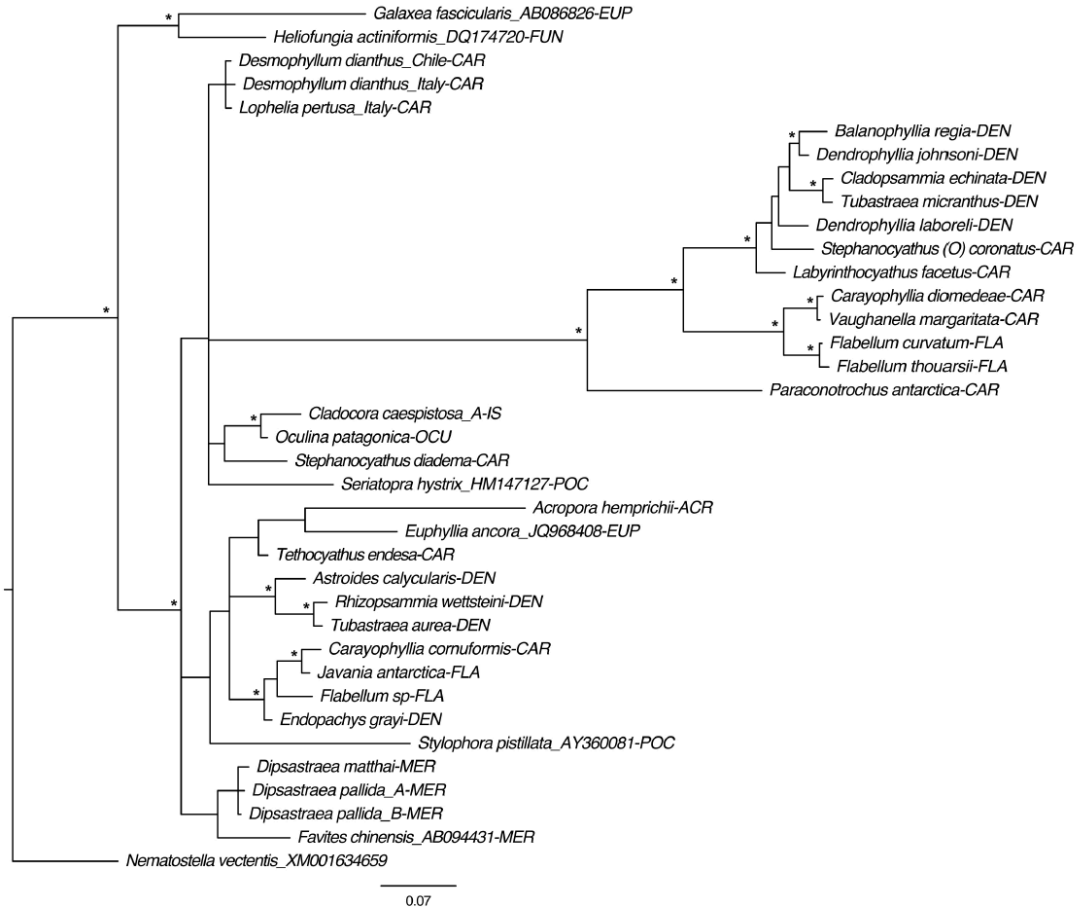

Figure 5.30. Phylogenetic reconstruction among scleractinian taxa based on COR10-ßActin. The relationship was inferred by BI and MP criteria, and asterisk (*) indicates well-supported node (pp ≥ 95; bootstrap > 70)

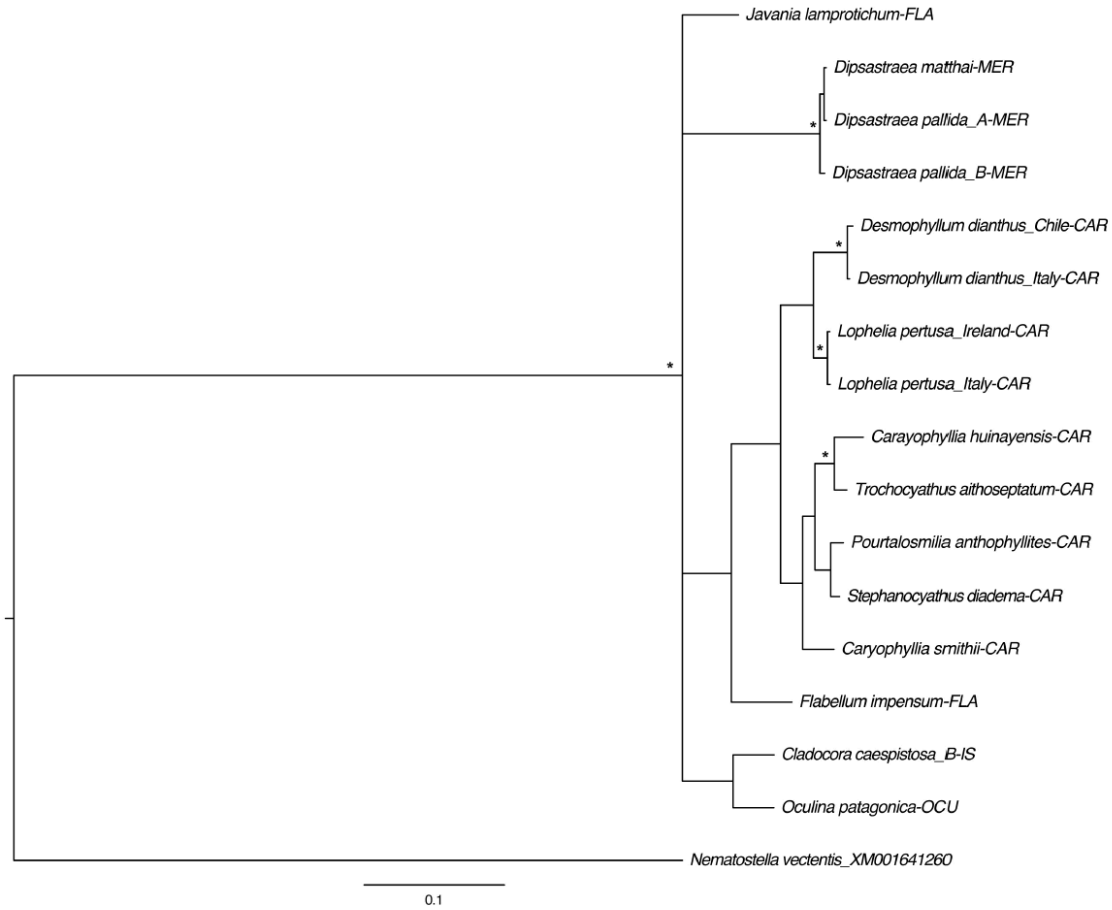

Figure 5.31. Phylogenetic reconstruction among scleractinian taxa based on COR12-Helicase. The relationship was inferred by BI and MP criteria, and asterisk (*) indicates well-supported node (pp ≥ 95; bootstrap > 70)

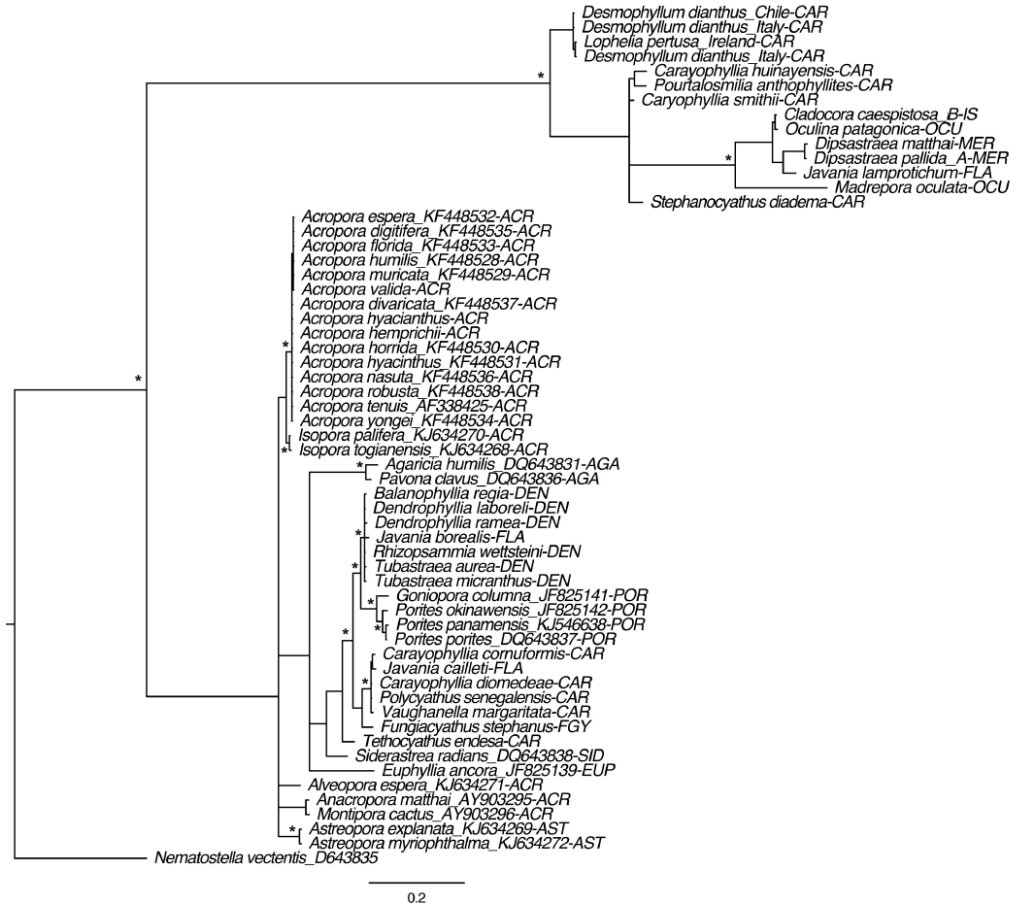

Figure 5.32. Phylogenetic reconstruction among scleractinian taxa based on COR14-NAD5. The relationship was inferred by BI and MP criteria, and asterisk (*) indicates well-supported node (pp ≥ 95; bootstrap > 70)

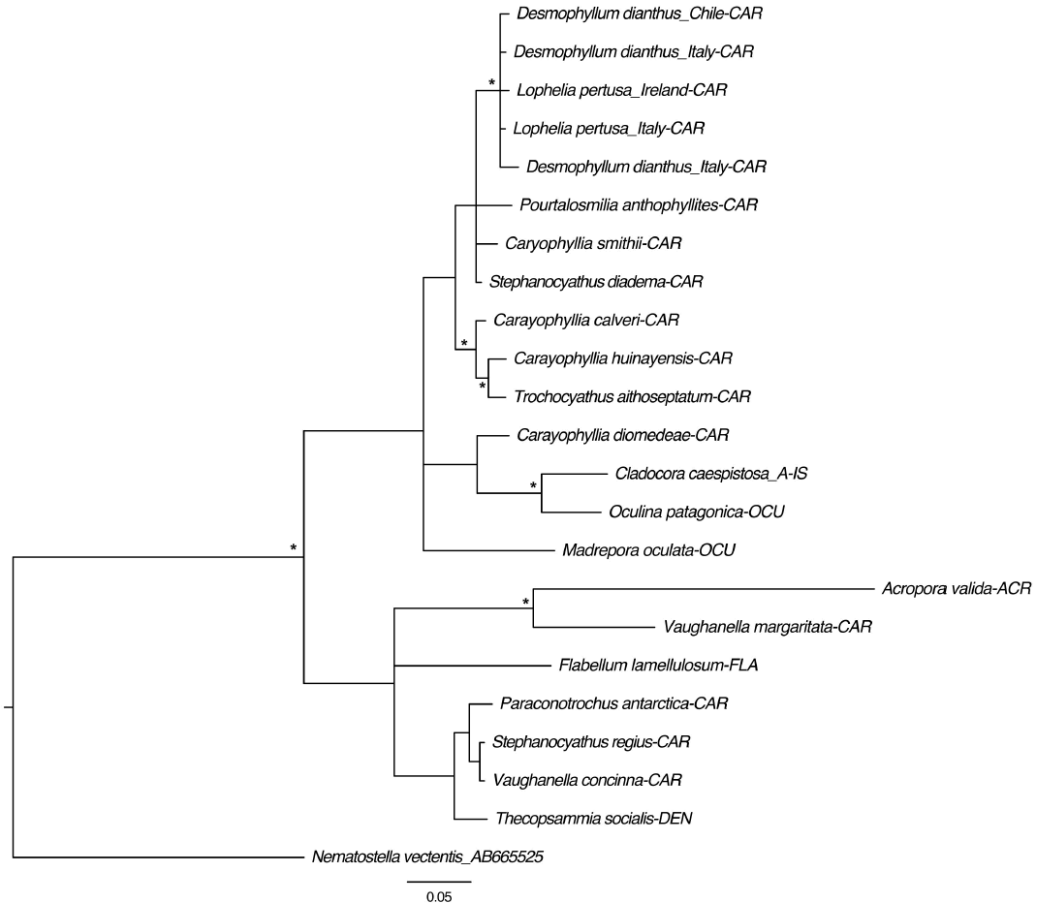

Figure 5.33. Phylogenetic reconstruction among scleractinian taxa based on COR15-Creatine kinase. The relationship was inferred by BI and MP criteria, and asterisk (*) indicates well-supported node (pp ≥ 95; bootstrap > 70)

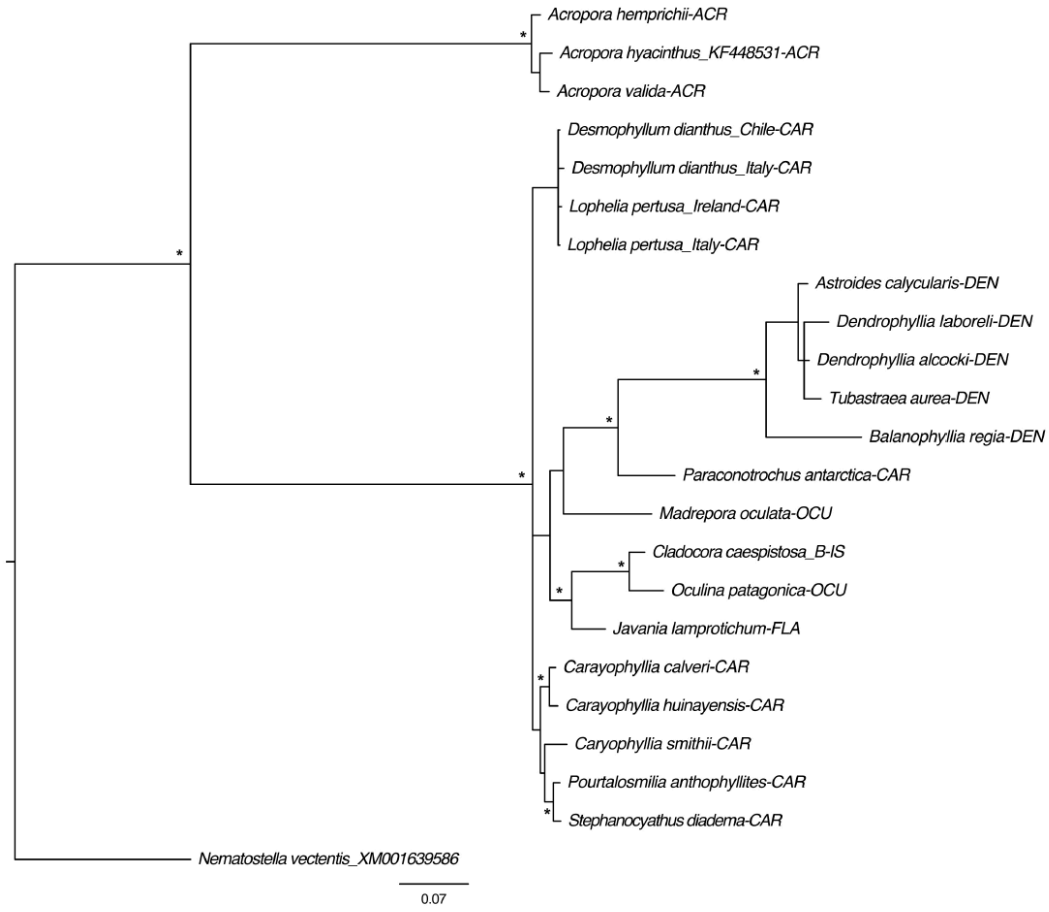

Figure 5.34. Phylogenetic reconstruction among scleractinian taxa based on COR17-NCAH like. The relationship was inferred by BI and MP criteria, and asterisk (*) indicates well-supported node (pp ≥ 95; bootstrap > 70)

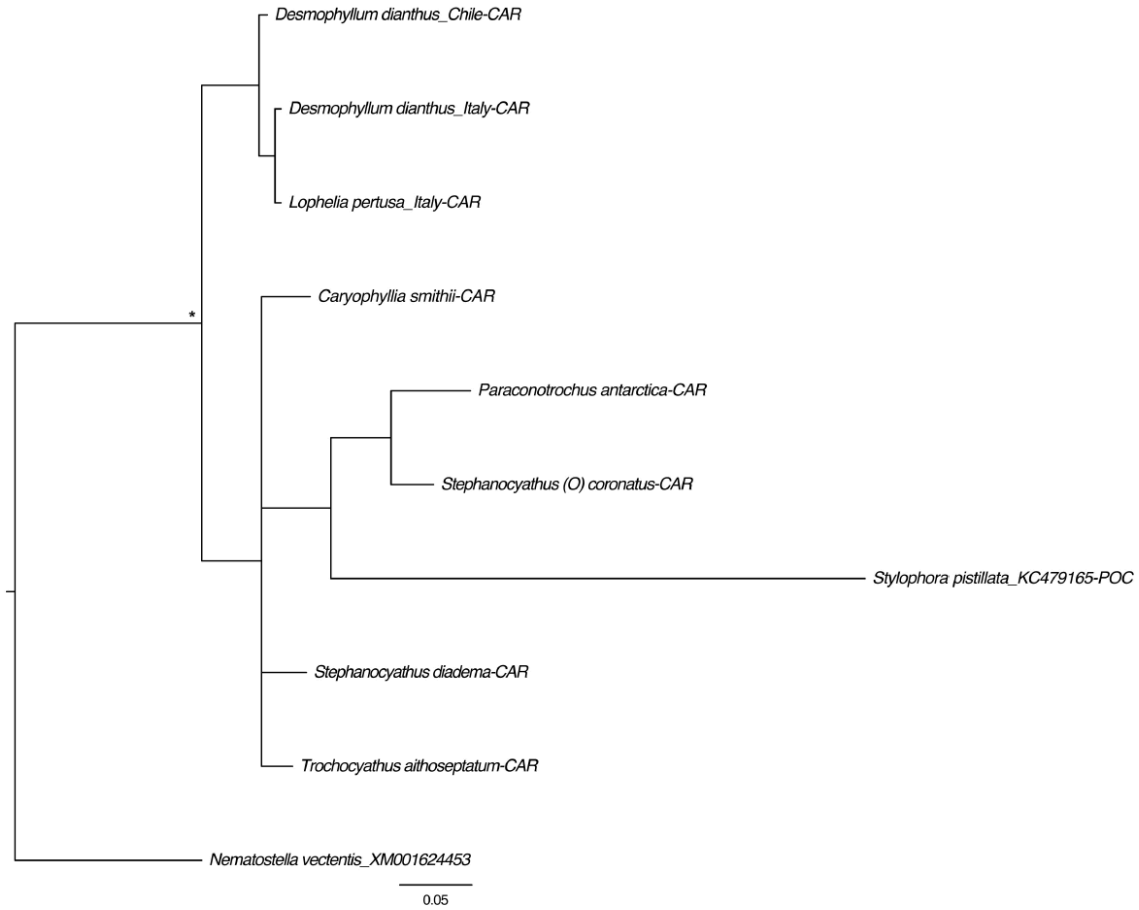

Figure 5.35. Phylogenetic reconstruction among scleractinian taxa based on COR21-UBB. The relationship was inferred by BI and MP criteria, and asterisk (*) indicates well-supported node (pp ≥ 95; bootstrap > 70)

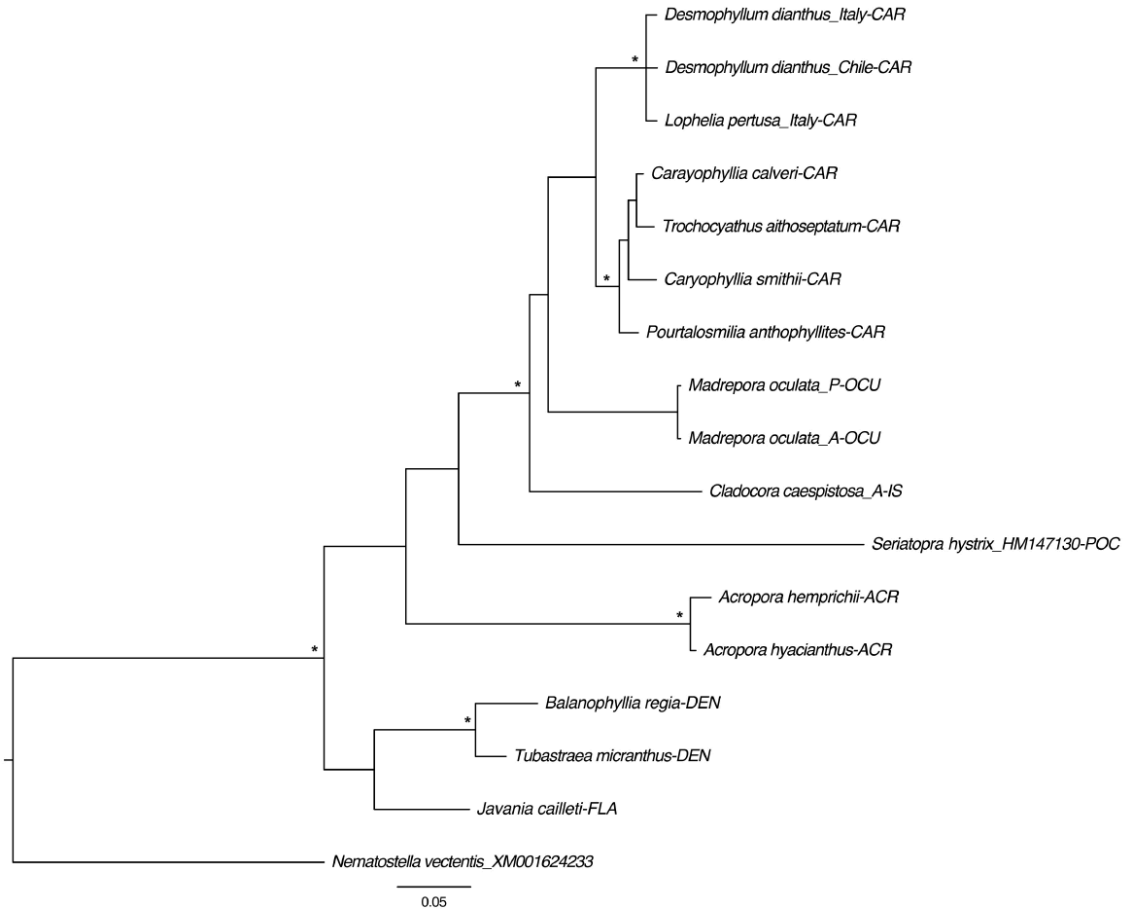

Figure 5.36. Phylogenetic reconstruction among scleractinian taxa based on COR24-Heat shock like. The relationship was inferred by BI and MP criteria, and asterisk (*) indicates well-supported node (pp ≥ 95; bootstrap > 70)

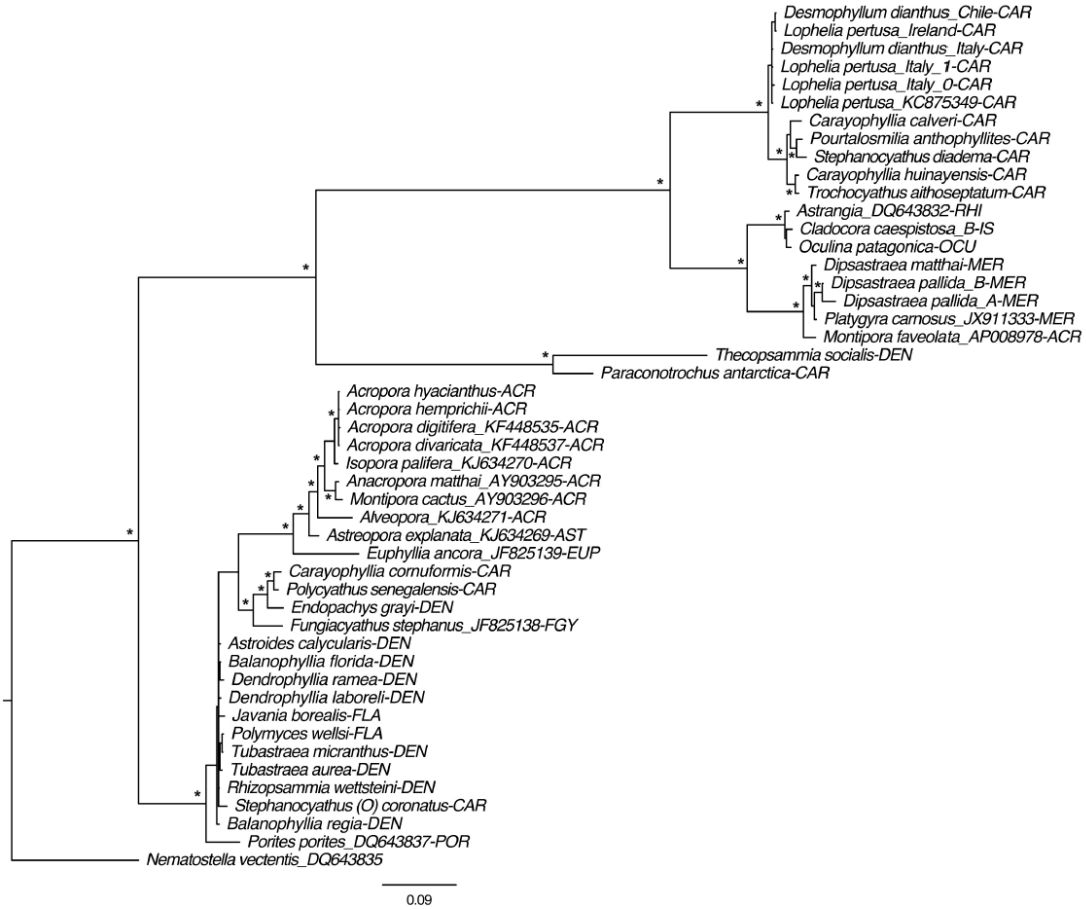

Figure 5.37. Phylogenetic reconstruction among scleractinian taxa based on COR25-16S rDNA. The relationship was inferred by BI and MP criteria, and asterisk (*) indicates well-supported node (pp ≥ 95; bootstrap > 70)

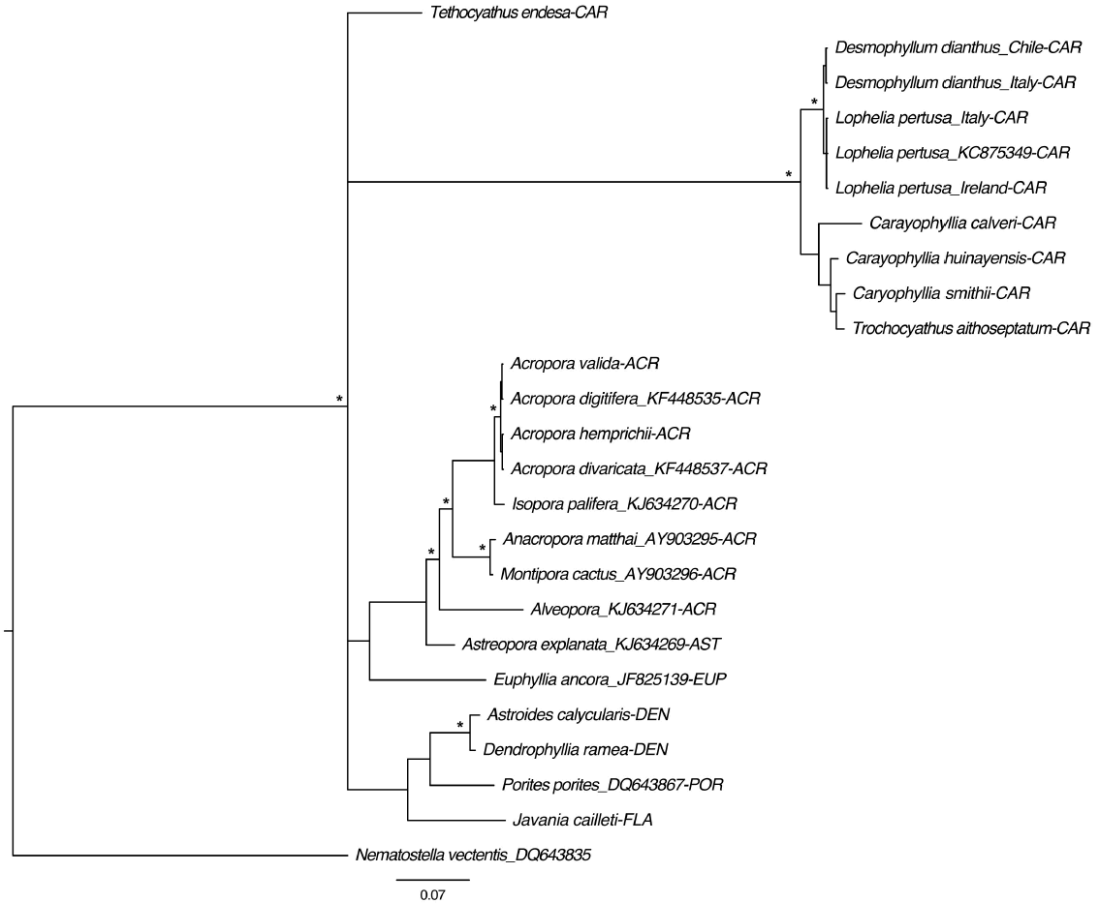

Figure 5.38. Phylogenetic reconstruction among scleractinian taxa based on COR26-ATP6NAD4. The relationship was inferred by BI and MP criteria, and asterisk (*) indicates well-supported node (pp ≥ 95; bootstrap > 70)

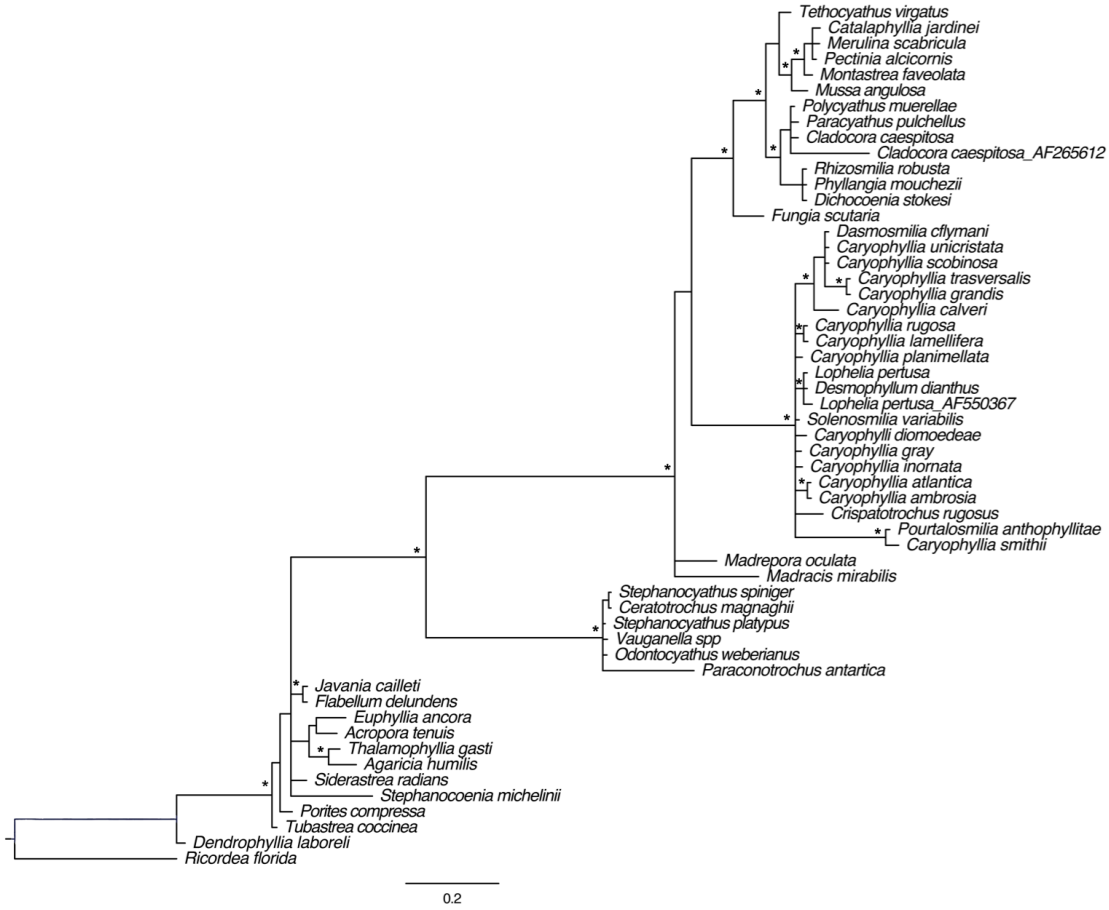

Figure 5.39. Phylogenetic reconstruction among scleractinian taxa based on 16s rDNA (Addamo et al 2012). The relationship was inferred by BI, ML and MP criteria, and asterisk (*) indicates well-supported node (pp ≥ 95; bootstrap > 70)

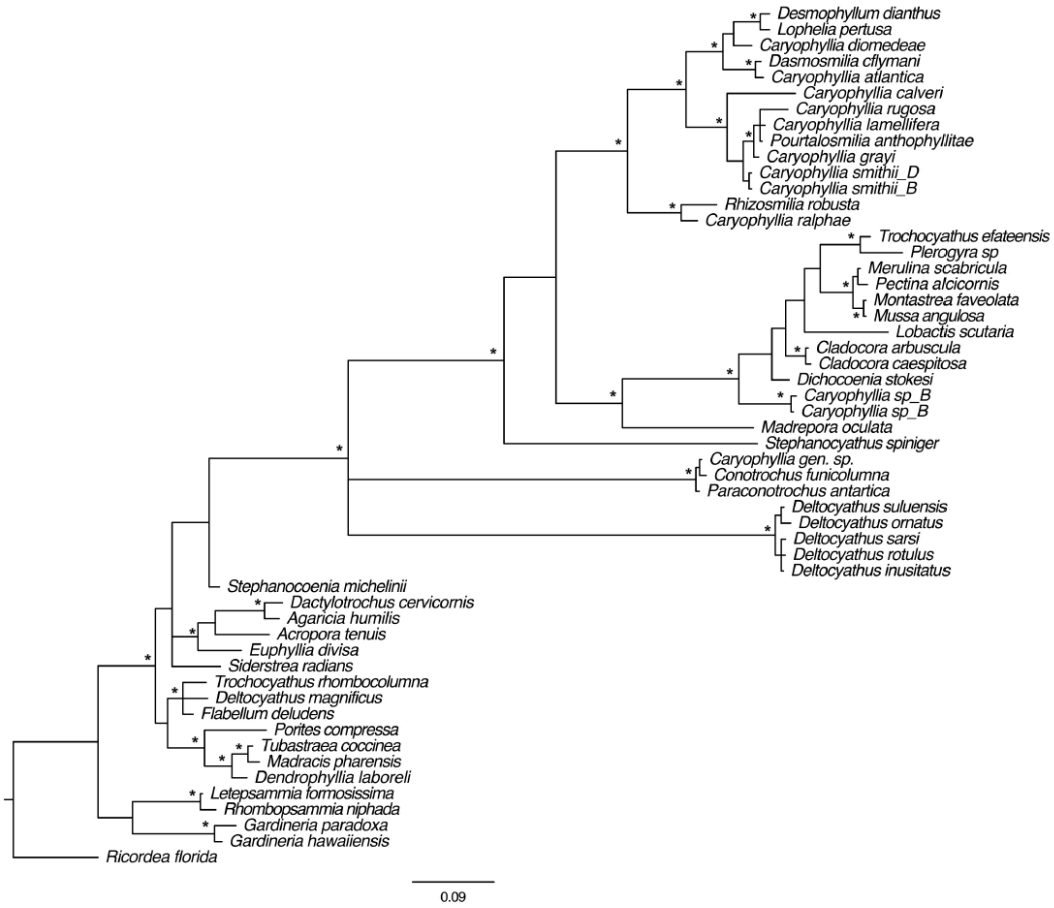

Figure 5.40. Phylogenetic reconstruction among scleractinian taxa based on COI (Addamo et al 2012). The relationship was inferred by BI, ML and MP criteria, and asterisk (*) indicates well-supported node (pp ≥ 95; bootstrap > 70)

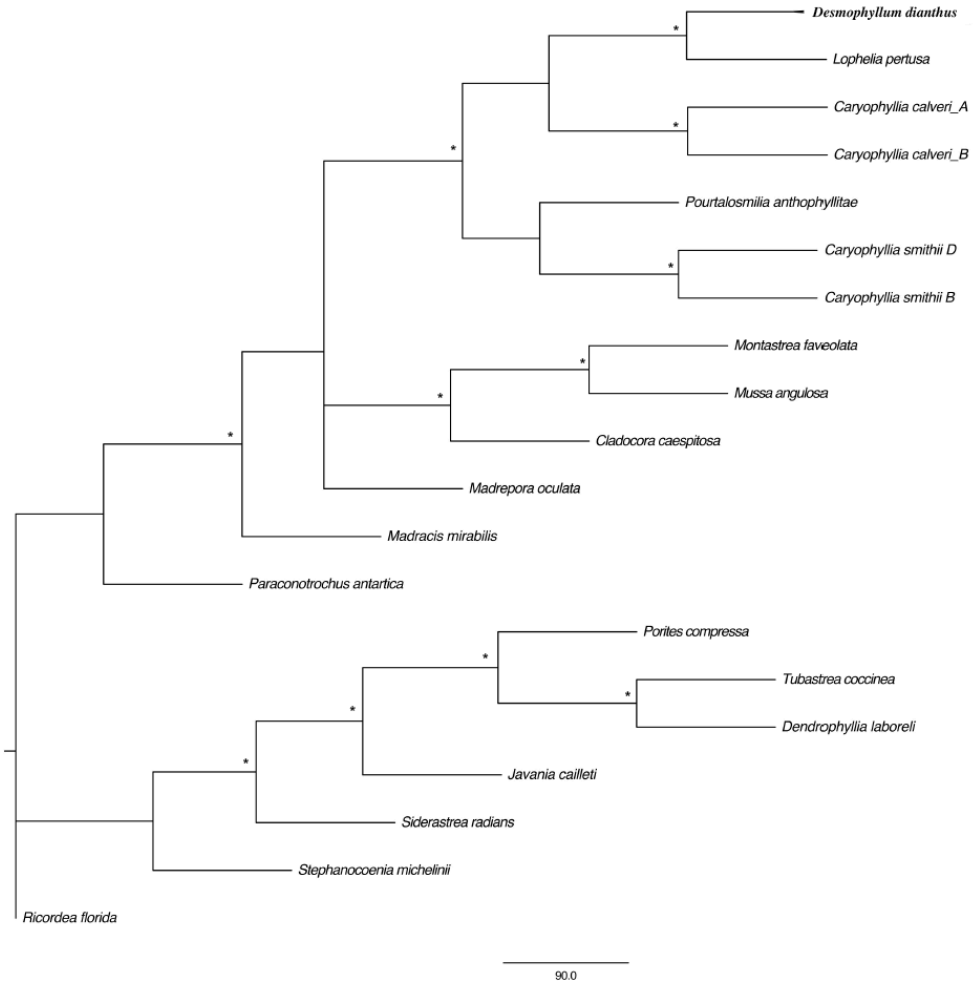

Figure 5.41. Phylogenetic reconstruction among scleractinian taxa based on concatenated mitochondrial (16S and COI) and nuclear genes (28S and ITS) (Addamo et al 2012). The relationship was inferred by BI, ML and MP criteria, and asterisk (*) indicates well-supported node (pp ≥ 95; bootstrap > 70)

Discussion

One of the most striking results is the lack of correlation between taxonomic level and divergence found for most of the markers; therefore, taxa defined as belongings to different genera or families showed a value of genetic distance equal to zero. On one hand, from a phenetic point of view, the high similarity and overlapping genetic divergence ranges between different families, genera and species, suggests that the revision of Scleractinia taxonomy is still an ongoing process, and lot of work needs to be done at any phylogenetic level. However, these results also pinpoint the necessity of being extremely cautious during the treatment of massive data, since the distancing of them, from the standpoint of automation, leads to spurious deductions. For instance, when hundreds or thousands of sequences are downloaded from databases and are automatically aligned, the researcher loses the possibility of controlling the entire amount of data. Thereafter, some figures obtained from the matrices are simply false. Even when two taxa are represented by sequences that do not overlap at all, we obtain a value of divergence. Such divergence has no meaning at all, but it is difficult to detect these cases if no careful survey of the raw data is performed.

On the other hand, from an a phylogenetic point of view in which a genetic distance is not enough for distinguishing different taxa, each gene analysed in this study showed homologous characters, whose ratio apomorphic/synapomorphic variables led to consider these novel genes as potential molecular markers for scleractinan barcoding and/or phylogeny studies. Results from the substitution saturation test demonstrated three types of pattern: A) group of markers where both Ti and Tv fall a linear correlation respect to the divergence, with Ti constantly outnumbering Tv, then the molecular markers have not experienced substitution saturation and are useful for phylogenetic analysis (Xia and Xie 2001). This is the case for COR4, COR 7, COR12, COR17 and COR24; B) group of markers where both Ti and Tv show a linear correlation, reaching a limit beyond which Tv have outnumbered Ti, experiencing full substitution saturation. These markers could be useful but, reached a threshold, their phylogenetic signal is questionable. Thus, they are little useful to reconstruct phylogenetic relationships at certain levels, because historical information about the nucleotide sequences has been overwritten a number of times (Xia *et al.* 2003). This is the case for 28S, CYTB and COR3; and finally C) group of markers where both Ti and

Tv fall in a plateau, reaching threshold beyond which they are not longer useful to phylogenetic analyses. This is the case of the most genes analysed, including those that are currently been used in phylogenetic studies of Scleractinia. Nevertheless, this plateau was here usually reached at very high levels of divergence, mostly greater than 30%. Thus, these markers might be probably useful at genera or species level. This point has to be evaluated using a proper taxa selection.

By the late 1980s, the debate between species trees and gene trees was intense and lead to an especially problematic question for closely related species (e.g. Tajima 1983; Nei 1987; Pamilo and Nei 1988). Pamilo and Nei (1988) suggested that combining information from several independent loci is better than adding more samples. Thus, building a species tree would require combining information from multiple genes, and all gene phylogenies need to be "embedded" inside the species history while not violating the species tree constraints: the time of a common ancestor of a gene cannot be more recent than the time of divergence of the respective species (Heled and Drummond 2010). In the last few years, several studies highlighted that multiple genes were extremely useful to resolve the systematics of many organisms (e.g plant, insect), for example, when comparative studies of traits variation remain limited by the lack of a well-supported phylogeny based on only one gene (Gibson and Baker 2012) or taxa (Pollock *et al.* 2002). Moreover, single copy nuclear genes resulted in being of immense value in plant systematics, providing regions with different phylogenetic signal deriving from coding and non-coding parts. Thus they can be applied to a wide range of taxa level: from families to intraspecific (Naumann *et al.* 2011).

Given all these considerations and analysing comparatively the phylogenetic reconstructions of each old and novel markers, it is consistent to state that all genes are carrying on their own evolutionary history and phylogenetic signal, even though strength could be variable at different taxonomic levels. Polyphyly of Caryophylliidae family has already been demonstrated with different markers in previous works (Kitahara *et al.* 2010b; Stolarski *et al.* 2011; Addamo *et al.* 2012) as well as with the new genes analysed in this study, suggesting that a complete and deep review of the family needs to be done. Closed relationship between *D. dianthus* and *L. pertusa*, is present in each phylogenetic reconstruction and continues to be congruent with previous work (Addamo *et al.* 2012). Furthermore, interesting results were obtained for *P.*

antarctica, a caryophylliid species with a taxonomic reassignment that was never 'confirmed' and/or analysed from a molecular point of view. Initially it was classified as a flabellid species *Gardineria antarctica* Gardiner, 1929, Contrary to opinions of Zibrowius (1974) and Cairns (1982), Cairns 'choose' to remove *G. antarctica* from the genus based on its morphological characters, more closely allied to the caryophyliid genera *Crispatotrochus*, *Conotrochus*, or *Labyrinthocyathus* (Cairns 1989). When the new genus *Paraconotrochus,* which was named after its resemblance and 'surmised' evolutionary proximity to *Conotrochus* Seguenza, 1864, was described, two species from genus *Gardineria* Vaughan, 1907 were 'tentatively' placed in the novel genus: *Duncania capensis* Gardiner, 1904 and *G. antarctica* Gardiner, 1929 (Cairns 1989; Cairns and Parker 1992). Results obtained in this study confirmed the hypothesised close phylogenetic relationships between *Paraconotrochus* and *Conotrochus,* and its taxonomic re-evaluation respect to *Gardineria* genera, which was found to be the 'basal group' of Scleractinia. Further and more exhaustive morphological analyses are been taken in consideration in order to support molecular results (P. Lopez, pers. comm.).

A similar revision should be considered for Dendrophylliidae and Flabellidae as well. In both cases, these families have been defined as monophyletic (Le Goff-Vitry *et al.* 2004; Fukami *et al.* 2008; Kitahara *et al.* 2010b; Arrigoni *et al.* 2014b), but results in this study, which included taxa that have not been analysed before from a molecular point of view (e.g. *Thecopsammia socialis*, *D. laborelli,* and *Endopachys grayi* for Dendrophylliidae; *Flabellum alabastrum*, *F. curvatum*, *F. thouarsii*, *Javania antarctica*, *J. borealis*, *J. lampotrichum* for Flabelliidae), suggest a fracture in this monophyletic pattern.

Other interesting outcomes are related to the closed phylogenetic relationship among *O. patagonica*, *Astrangia* sp. and *C. caespitose*; species that have been defined as belonging to Oculinidae, Rhizangiidae, and *incertae sedis* respectively. The latter species in particular has had a tangled taxonomic history: initially the genus was considered within Faviidae (Veron 1995) family, but based on a preliminary molecular study and a revision of the morphological characters, the dubious affiliation was later changed for Caryophylliidae (Romano and Cairns 2000; Cairns *et al.* 2001). Subsequently, results from a more exhaustive molecular phylogeny reconstruction of Scleractinia suggested it to be included in Oculinidae (Fukami *et al.* 2008). Recently the

species has been moved into an undefined group of corals (Hoeksema 2014). Results from this and previous studies (Kitahara *et al.* 2010b; Addamo *et al.* 2012) not only demonstrated *O. patagonica* and *C. caespitosa* consistently clustered in a well supported clade, but also that it was not closely related to the oculiniid species *M. oculata*. Said results can also contribute to the complete taxonomic revision of all these species/families that has already been taken in consideration at molecular and morphological level (F. Benzoni, pers. comm.).

This study presents a clear critical point related to the use of specimens of museum collection and variable number of species used per each molecular marker. The preservation of Museum samples, their fixation and posterior conservation, lead to different states of DNA integrity. Thus, it was not always possible, with all the markers tested, to amplify or obtain adequate sequences quality to have a complete data set. Moreover, the design of the primers used could be also a turning point, since they were prepared upon the sequences of seven different taxa and their "universality" might be dubious then. Although all these factors do not allow accurate inferences about the phylogenetic relationships among taxa, the consistent results are not only obtained from different phylogenetic reconstruction methods, but also from different genes, which are carrying on their own evolutionary history, which coincided for phylogenetic relationships between taxa, giving evidences of truly phylogenetic resolution. Therefore, combining results from genetic divergence calculation, substitution saturation test and phylogenetic reconstruction, novel markers are classified in three main groups of markers potentially useful at the following phylogenetic levels: A) at family and/or higher taxonomic level: COR6 and COR7; B) at family level: COR3, COR14, COR25, and COR26; C) at family and genus level: COR2, COR4, and COR17. In addition, due to the low number of taxa analysed, further studies are needed in order to confirm the potential usefulness of the following novel markers: COR12 at family level, COR15 and COR21 at family-genus level; and COR10 at species level.

It is relevant to highlight the importance of finding useful 'A' group markers for high taxonomic levels. Indeed, they may be helpful to tackle the controversy about the phylogenetic relationships between Scleractinia and Corallimorpharia orders within Anthozoa, which are distinguished by the presence of an aragonite skeleton in the former (Medina *et al.* 2006; Kitahara *et al.* 2014; Lin *et al.* 2014). However,

scleractinian paraphyly ('naked corals' topology), based on the analysis of the sequences of proteins encoded in the mitochondrial genomes (Lin *et al.* 2014), is contradicted by phylogenetic studies based on mitochondrial nucleotide sequence data (Kitahara *et al.* 2014; Lin *et al.* 2014). Thus the 'naked coral' topology could be caused by high levels of saturation in these mitochondrial sequences, long-branch attraction or model violations. The equivocal results of these extensive analyses highlight the fundamental problems of basing coral phylogeny only on mitochondrial sequence data (Kitahara *et al.* 2014).

Trying to answer to the inevitable demand of useful molecular markers, to clarify the systematics in the Scleractinia order, could be a 'vicious circle'. Although this study provided potential tools for systematic study of Scleractinia at different taxonomic levels, it is important to highlight that the taxonomic level corresponding to each new marker has been determined upon a low number of taxa and, moreover, it is strictly related to the currently accepted scleractinian systematics. Clearly, once the Scleractinia taxonomy changes, then the phylogenetic potential of each marker will be clearly affected and this level of utility should be reconsidered.

Many recent studies are "shaking up" the coral taxonomy and systematics (Arrigoni *et al.* 2012; Kitano *et al.* 2013; Arrigoni *et al.* 2014a; Arrigoni *et al.* 2014b; Huang *et al.* 2014a; Huang *et al.* 2014b; Kitano *et al.* 2014). Large effort was employed for searching new morphological characters useful ftocoral taxonomy (Carlon and Budd 2002; Budd and Stolarski 2009; Benzoni *et al.* 2010; Kongjandtre *et al.* 2012), and their integration with molecular markers allowed taxonomists to create new families and genera, clarifying part of the systematics of Scleractinia. Nevertheless, most of the studies have focused their attention on shallow water corals and rarely included azooxanthellate species. Moreover, the molecular markers commonly used so far were not useful for all families and/or genera, showing contrast efficiency in their phylogenetic signal. So far, contrary to a morphologic point of view, any effort has been done to search new molecular markers that could be useful for coral phylogeny. From the results obtained in this study it is clear that it is extremely important to extend the taxa analysed to azooxanthellate corals and to apply new molecular markers in order to clarify the phylogeny of the Scleractinia order. Taking each of these results into account re-enforces the conclusion that increased sampling of taxa is one of the most important

ways to increase the overall phylogenetic accuracy (Pollock *et al.* 2002). Probably the new era of massive sequencing will help bringing the necessary data that will add the imperative knowledge to disentangle the currently 'messy' scleractinian taxonomy. This study is trying to start towards such promising way.

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CHAPTER VI

Chapter adapted from:

Addamo AM, Vertino A, Stolarski J, Garcia R, Taviani M, Machordom A (In prep.) Going against the current: the overwhelming genetic similarity between solitary and colonial corals: *D. dianthus* versus *L. pertusa*.

Desmophyllum fasciculatum. Joubin L (1928). Note sur un coralliaire du genre *Desmophyllum* Bulletin du Muséum National d'Histoire Naturelle.

Going against the current: the overwhelming genetic similarity between solitary and colonial corals: *D. dianthus* versus *L. pertusa*.

Abstract

Despite several types of molecular markers were demonstrated to be powerful tools in Systematics, the species concept and taxonomic problems with closely related species are still one of the most complex issues that taxonomists have to face in many groups of organisms, such as Scleractinia. It is a taxonomically complex order that make this problem even more difficult to disentangle. Comparative analyses between *Desmohyllum dianthus*, a solitary coral, and *Lophelia pertusa*, a colony coral, were performed using newly sequenced complete mitochondrial genomes, 30 microsatellites and other 18 molecular markers, including protein-coding and non-coding genes - previously published - in order to define the genetic divergence between this two genera. Results suggested that taxonomic classification of both species should be reconsidered at both genus and species level.

Keywords: mitochondrial genome, microsatellites, molecular markers, genetic divergence, *Lophelia pertusa*, *Desmophyllum dianthus.*

Introduction

Mitogenomics, or whole-genome mitochondrial DNA data set, revealed to be powerful phylogenetic tools in a wide range of organisms, improving phylogenetic estimations, reconstructing robust phylogenies and resolving long-standing phylogenetic uncertainties (Curole and Kocher 1999; Irisarri *et al.* 2010; Duchêne *et al.* 2011; Vilstrup *et al.* 2011; Weisrock 2012; Osca *et al.* 2014). Although the coral mitochondrial genome was estimated to be evolving 10-20 times and 5 times more slowly than vertebrate and nuclear scleractinian counterpart respectively (van Oppen *et al.* 2002; Chen *et al.* 2009), its limited application to species phylogeny and population genetics has been suggested (Shearer *et al.* 2002; Hellberg 2006). Exceptions to the

mentioned applicable limitation were demonstrated in population genetics, where it was reported as useful to detect population variability and structure (Watanabe *et al.* 2005; Chen *et al.* 2008). Furthermore, mitochondrial genome rearrangements occur relatively infrequently and have revealed to be useful for bringing in sight unexpected evolutionary relationships, resolving closely related species particularly in Scleractinia (Fukami and Knowlton 2005; Flot and Tillier 2007; Emblem *et al.* 2011; Lin *et al.* 2011; Lin *et al.* 2012; Kitahara *et al.* 2014; Lin *et al.* 2014).

Desmophyllum dianthus and *Lophelia pertusa* are azooxanthellate scleractinian corals, two of the few cosmopolitan species of Scleractinia, distributed throughout the world oceans except in polar seas (Zibrowius 1980; Cairns 1982). They are common on deep-waters, where *D. dianthus*, as a solitary coral, is associated with main framework building species, as the colonial coral *L. pertusa* (Rogers 1999). Although both species are usually forming deep-water reefs on continental slope, mid-oceanic ridges and fjords (Rogers 1999), exceptional shallowest records were reported for *D. dianthus* and *L. pertusa* in Chilean (8 m) and Norwegian fjords (39 m) respectively (Rapp and Sneli 1999; Försterra and Häussermann 2003). Besides the ecologically sympatric relationship (sharing the same habitat), genetic similarity was also reported between *Desmophyllum* and *Lophelia* in previous genetic analyses (Addamo *et al.* 2012), suggesting an ambiguous taxonomic status that required to be confirmed.

The objective of this study is to performe comparative genetic analyses, establishing genetic fingerprint and phylogenetic relationship between *D. dianthus* and *L. pertusa*. New data of complete mitochondrial genome and other molecular markers including protein-coding and non-coding genes, previously analysed, were used in order to reach an exhaustive interpretation.

Material and Methods

Samples collection and study area

For amplification of the complete mitochondrial genome, two samples of *D. dianthus* were collected in two distant localities: 1) South Adriatic Sea (39º53’468’’N, 18º55’176’’E), located in Tricase off shore (Italy, Mediterranean Sea) and sampled at

786 m depth by R/V *Urania* during CNR cruise MEMA12 in April-May 2012; and 2) Isla Jaime, (43º46'34.23''S, 72º55'13.057''W), located in Pitipalena Fjord (Chile, South Pacific Ocean) and sampled at 23 m depth by SCUBA diving in February 2012.

For additional sequence comparison of putative control region, 13 samples of *D. dianthus* and 2 of *L. pertusa* were collected in 15 distinct localities distributed in both northern and southern hemispheres. Complete information related to the above mentioned specimens could be found in Table 6.1.

Specimens here analysed were preserved in absolute ethanol. All necessary permits were obtained for the described field studies and samples were transported to Spain with appropriate export and import permits following the Convention on International Trade in Endangered Species of wild Fauna and Flora (CITES). This study did not involve endangered or protected species listed in the IUCN Red List of Threatened Species.

Table 6.1. Depth, technique, geographic coordinates and others information of sampling localities

Codigo MNCN	EXPEDITION	Country	Province/State	Precise Locality	Range Depth	Lat (start)	Long (start)	Depth (start)	Lat (end)	Long (end)	Depth (end)	Technique	Date (M/A)
DdADR_635	MEMA12	Italy	South Adriatic	off shore Tricase	786	39°53'468"N	18°55'176"E					Grab	Apr-May 2012
DdADR_636	MEMA12	Italy	South Adriatic	off shore Tricase	786	39°53'468"N	18°55'176"E					Grab	Apr-May 2012
DdARG_476	Patagonia 0209	Argentina	off Argentina/ Falkland Islands	off Argentina/ Falkland Islands	1244	43°17'5.64"S	59°0'3.24"W					Trawl	Feb 2009
DdAUS_566	SS02/2007	Australia	Tasmania	Dory Hill W	1100-1200	44°19'34.536"S	147°7'8.148"E	1100	44°19'33.816"S	147°6'50.436"E	1200	Gear Sled	Apr 2007
DdAVI_656	INDEMARES 0710	Spain	Cantabria	Cañon de Áviles	500	43°43'44.76"N	6°5'24.359"W	500				Grab, rock sled, epibenthic sleds	July-Aug 2010
DdIJC_432		Chile	Pitipalena fijord-Region XI	Isla Jaime	23	43°46'27"S	72°55'045"W					SCUBA	Feb 2012
DdIJC_433		Chile	Pitipalena fijord-Region XI	Isla Jaime	23	43°46'27"S	72°55'045"W					SCUBA	Feb 2012
DdILC_681		Chile	Region X	Isla Lilihuapi - Comau fijord	20	42° 9'43.82"S	72°35'54.22"W					SCUBA	Aug 2012
DdIRL_480	EUROFLEETS-CWC Moira	Ireland	off Cork-Galway	Moira Mounds	1069	51°26'33"N	11°49'38"W					Box Corer	June 2012
DdMNZ_601	TAN0803	New Zealand	Macquaire Ridge	Macquaire Ridge	385	51°3'39.6"S	161°28'40.8"E					Grab, rock sled, epibenthic sleds	Apr 2008
DdSML_62	CORSARO 39	Italy	South Adriatic	off Santa Maria di Leuca	577-540	39°33'14.8"N	18°13'16.3"E	577	39°33'27.4"N	18°13'111"E	540	Epibenthic dredge	Apr 2006
DdSML_72	CORSARO 39	Italy	South Adriatic	off Santa Maria di Leuca	577-540	39°33'14.8"N	18°13'16.3"E	577	39°33'27.4"N	18°13'111"E	540	Epibenthic dredge	Apr 2006
Lp272	CORSARO 39	Italy	South Adriatic	off Santa Maria di Leuca	577-540	39°33'14.8"N	18°13'16.3"E	577	39°33'27.4"N	18°13'111"E	540	Epibenthic dredge	Apr 2006
Lp296	EUROFLEETS-CWC Moira	Ireland	off Cork-Galway	Moira Mounds	1069	51°26'33"N	11°49'38"W					Box Corer	June 2012

DNA extraction and mitochondrial genome sequencing

Genomic DNA was extracted from the mesenteric tissue of each specimen using the QIAGEN BioSprint 15 DNA Blood Kit (Qiagen Iberia S.L., Madrid, Spain), with slight modifications, including the optional RNase treatment and an extended period of proteinase K lysis (overnight incubation at 55 °C). DNA concentration was quantified using the Qubit 2.0 Fluorometer and diluted to a final concentration of 2 ng/μl.

Several overlapping fragments covering the whole mitogenome were amplified by PCR using the primers previously designed for *L. pertusa* (Flot *et al.* 2013). One specific primer pair was designed by the authors using PRIMER3 (Rozen and Skaletsky 2000) (Table 2). PCRs were carried out in a total volume of 50 μl with 1x PCR Biotools Standard Reaction Buffer including 2 mM $MgCl_2$, 0.5 mM forward and reverse primers, 0.2 mM of each dNTP, 1.5U DNA polymerase (Biotools), and 2 ng of template DNA. PCR amplifications were performed in a Veriti^{TM} Thermal Cycler (Applied Biosystems) with the following cycles conditions: an initial denaturing step of 94 ºC for 5 min, followed by 35 cycles of 30 s at 94 ºC, an annealing step of 30 s at 53 ºC, an extension step of 1-3 min at 72 ºC, and a final extension of 10 min at 72 ºC. PCR products were purified using GELase^{TM} Agarose Gel-Digesting Preparation (Epicentre, Madison, WI, USA), following the Fast Protocol. When this profile did not amplify a specific PCR product, three other annealing temperatures (T_A 50, 51 or 53 ºC) were tested with the same cycling conditions. Nevertheless, when repeated attempts to amplified specific fragments failed, PCR amplifications were carried out in a total volume of 20 μl with the same conditions cited before but with 2U DNA polymerase (MyTaq), and 2 ng of template DNA. In theses cases, PCR amplifications were performed with the following cycles conditions: an initial denaturing step of 95 ºC for 5 min, followed by 40 cycles of 15 s at 95 ºC, an annealing step of 30 s at 50 ºC, an extension step of 1-3 min at 72 ºC, and a final extension of 10 min at 72 ºC.

To amplify the putative control region, specific PCR amplifications were performed in total volume of 20 μl with 1x PCR OptiBuffer Reaction Buffer including 3 mM $MgCl_2$, 1x Hi-Spec Additive, 0.5 mM forward and reverse primers, 0.5 mM of each dNTP, 2U DNA polymerase (BIO-X-ACT Short), and 2 ng of template DNA. PCR amplifications were performed with the following cycle conditions: an initial denaturing step of 95 ºC

for 5 min, followed by 30 cycles of 30 s at 94 ºC, an annealing step of 30 s at 56 ºC, an extension step of 2 min at 72 ºC, and a final extension of 10 min at 72 ºC.

PCR products were cloned into pGEM-T vectors (Promega, Madison, WI, USA), were purified using Wizard® *Plus* SV Minipreps DNA Purification System (Promega, Madison, WI, USA), following Centrifugation Protocol, and were sequenced using M13 universal primers.

Amplicons were sequenced using an ABI PRISM 3730 DNA Sequencer (Applied Biosystems), following Poly-A/T Protocol (Secugen S.L.) using specific primers and, when needed, primers walking designed to cover the total length of fragments (Table 6.2).

The complete mitogenomes and sequences reported in this paper will be deposited in NCBI GenBank.

Table 6.2. Primers pair used for amplification and sequencing. PW=primer walking

Oligo name	Oligo sequence (5' to 3')	Fragment length (bp)	Reference
LD1F	AAATCAAACGAGATTCCGAGAG	1198	Flot et al. 2013
LD1R	TCCATGGGGACTTCTCGTC		Flot et al. 2013
LD2F	TCGACTGTTTACCAAAAACATAGC	1519	Flot et al. 2013
LD2R	AAYAACCTTCCATTGCATCC		Flot et al. 2013
LD3F	TAGGAGTGGTTGGGAAATCG	2563	Flot et al. 2013
LD3R	CTTGGGGAAGCCAAATATGA		Flot et al. 2013
LD4F	GAACAACAGGGGCAACAGAT	2127	Flot et al. 2013
LD4R	ATGGTGTCCCTGAAAAGTCG		Flot et al. 2013
LD5F	GCAGACGCGGTGAAACTTA	2521	Flot et al. 2013
LD5R	TACCCCGGCTAAGACAACTG		Flot et al. 2013
LD6F	TTGTGGGGCAAATCATTCTT	1034	Flot et al. 2013
LD6R	AATGAGAAAGCCCACAAGCA		Flot et al. 2013
LD7F	CAACTCCGGTTTCTGCCTTA	3060	Flot et al. 2013
LD7R	TTTAAAAGAAAACTATGGAGGCCTAA		Flot et al. 2013
LD8F	TTATTGGGCCTGTGTTTGGT	1604	Flot et al. 2013
LD8R	CCCACATATGAAAAGGAGCAAC		Flot et al. 2013
LD9F	TGGGTGCTCTTTCTTCTGGT	1237	Flot et al. 2013
LD9R	AAATCCAATTGGTATATAATTTGTCA		Flot et al. 2013
LD10F	ATCCCTCCTTTTGCAGGATT	868	Flot et al. 2013
LD10R	CCCCAGAAGCTGTTGTGTTT		Flot et al. 2013
LD11F	GGCAATTGGTTCTGGGATAA	1254	Flot et al. 2013
LD11R	AAGCATACTAAAAGCCGTTCCA		Flot et al. 2013
LD12F	TCTACAAACCACAAAGATATCGG	930	This study
LD12R	AATCCCCGTAGGAACAGCAA		This study
LD13F	GCCGGTGCTATTACAATGCT	1892	Flot et al. 2013
LD13R	CAATCGATTCAAGCTCTTTTCA		Flot et al. 2013
1a.PWF	CCATGTCCCACGGTTTATGT		This study
1b.PWR	AGGCCCAACTAACCTTCCAT		This study
2a.PWF	CATGGCGATTTCTTCTGTGA		This study
2b.PWR	CCCCGTCACACTTATGATCC		This study
3.PWF	GAAGCTTTTGTCATGCTTCCTT		This study
4a.PWF	TGTGGAGTTTTCTCCTTGACC		This study
4b.PWR	AAGCTAACGTCTCGCCTTCA		This study
5a.PWF	GGTTGTGGCTTGTGGTCTTT		This study
5b.PWR	GCCCTCAAGGCAAAACATAA		This study
6a.PWF	ACAGTCGGGGCAAGTTTTTA		This study
6b.PWR	ACCAAACACAGGCCCAATAA		This study

Sequences alignment, annotation and analyses

Sequence chromatograms were verified and primers removed using Sequencher v.4.10.1 (Gene-Code Corporation). The genome sequences were confirmed using the NCBI BLAST program and assembled using Sequencher v4.10.1, and were subsequently compared with three mitogenomes of *L. pertusa* previously published (Emblem *et al.* 2011; Flot *et al.* 2013) (Table 6.3).

Examination of open reading frames (ORFs) was performed using ORF Finder (available online at http://www.bioinformatics.org/sms2/orf_find.html), setting searching parameters at codon length > 50 amino acids, and with the Coelenterate Mitochondrial Code translation. Transfer RNA genes were identified using tRNAscan-SE 1.21 (Lowe and Eddy 1997) (available online at http://lowelab.ucsc.edu/tRNAscan-SE/). Additional automatic annotations were performed with DOGMA (Wyman *et al.* 2004) (available online at http://dogma.ccbb.utexas.edu/) using high COVE threshold for mitochondrial tRNAs (= 30), and MITOS (Bernt *et al.* 2013) (available online at http://mitos.bioinf.uni-leipzig.de/index.py). Mitochondrial protein-coding genes of *L. pertusa* and *D. dianthus* were compared to calculate nonsynonymous (dN) and synonymous (dS) substitution rates through model selection and model averaging, using three different methods based on Maximum-Likelihood and implemented in KaKs_Calculator (Zhang *et al.* 2006).

To compare the genome in a larger range of species, 50 mitogenomes of corals, representing 5 families and 15 genera of the Scleractinia Order previously published, were also retrieved from NCBI GeneBank and aligned in ClustalW using the default setting (Larkin *et al.* 2007) (Table 6.3). The resulting alignments were manually checked and adjusted with Se-Al v.2.0a11 (Rambaut 2002). Estimation of genetic divergence between pairs of taxa, using uncorrected p-distances were calculated in PAUP*v4.0a134 (Swofford 2002). To estimate genetic divergence among genera and families of corals, mean uncorrected p-distances were calculated in Sequencer 6.1 (shareware written by B. Kessing and available at: http://nmg.si.edu/sequencer/).

Table 6.3. List of mitochondrial genome used with GenBanK Accession Numbers (update July 2014)

Family	Genus	Species	bp	GenBank Acc. No.
Acroporidae	*Acropora*	*aspera*	18,479	KF448532
Acroporidae	*Acropora*	*digitifera*	18,479	KF448535
Acroporidae	*Acropora*	*divaricata*	18,481	KF448537
Acroporidae	*Acropora*	*florida*	18,365	KF448533
Acroporidae	*Acropora*	*horrida*	18,480	KF448530
Acroporidae	*Acropora*	*humilis*	18,479	KF448528
Acroporidae	*Acropora*	*hyacinthus*	18,566	KF448531
Acroporidae	*Acropora*	*muricata*	18,481	KF448529
Acroporidae	*Acropora*	*nasuta*	18,481	KF448536
Acroporidae	*Acropora*	*robusta*	18,480	KF448538
Acroporidae	*Acropora*	*tenuis*	18,338	AF338425
Acroporidae	*Acropora*	*yongei*	18,342	KF448534
Acroporidae	*Alveopora*	*sp.*	18,146	KJ634271
Acroporidae	*Anacropora*	*matthai*	17,888	AY903295
Acroporidae	*Astreopora*	*explanata*	18,106	KJ634269
Acroporidae	*Astreopora*	*myriophthalma*	18,106	KJ634272
Acroporidae	*Isopora*	*palifera*	18,725	KJ634270
Acroporidae	*Isopora*	*togianensis*	18,637	KJ634268
Acroporidae	*Montipora*	*cactus*	17,887	AY903296
Agariciidae	*Agaricia*	*humilis*	18,735	DQ643831
Agariciidae	*Pavona*	*clavus*	18,315	DQ643836
Astrocoenidae	*Madracis*	*mirabilis*	16,951	EU400212
Caryophylliidae	*Lophelia*	*pertusa*	16,150	NC_015143
Caryophylliidae	*Lophelia*	*pertusa* 302	16,149	KC875348
Caryophylliidae	*Lophelia*	*pertusa* 362	16149	KC875349
Caryophylliidae	*Polycyathus*	*sp.*	15,357	JF825140
Euphylliidae	*Euphyllia*	*ancora*	18,875	JF825139
Fungiacyathidae	*Fungiacyathus*	*stephanus*	19,381	JF825138
Merulinidae	*Montastraea*	*annularis*	16,138	AP008973
Merulinidae	*Montastraea*	*annularis*	16,138	AP008974
Merulinidae	*Montastraea*	*faveolata*	16,138	AP008977
Merulinidae	*Montastraea*	*faveolata*	16,138	AP008978
Merulinidae	*Montastraea*	*franksi*	16,138	AP008975
Merulinidae	*Montastraea*	*franksi*	16,137	AP008976
Merulinidae	*Platygyra*	*carnosus*	16,463	JX911333
Mussidae	*Colpophyllia*	*natans*	16,906	DQ643833
Mussidae	*Mussa*	*angulosa*	17,245	DQ643834
Oculinidae	*Madrepora*	*oculata*	15,841	JX236041
Pocilloporidae	*Pocillopora*	*damicornis*	17,415	EF526302
Pocilloporidae	*Pocillopora*	*damicornis*	17,425	EU400213
Pocilloporidae	*Pocillopora*	*eydouxi*	17,422	EF526303
Pocilloporidae	*Seriatopora*	*caliendrum*	17,010	EF633601
Pocilloporidae	*Seriatopora*	*hystrix*	17,059	EF633600
Pocilloporidae	*Stylophora*	*pistillata*	17,177	EU400214
Poritidae	*Goniopora*	*columna*	18,766	JF825141
Poritidae	*Porites*	*okinawensis*	18,647	JF825142
Poritidae	*Porites*	*panamensis*	18,628	KJ546638
Poritidae	*Porites*	*porites*	18,648	DQ643837
Rhizangiidae	*Astrangia*	*sp.*	14,853	DQ643832
Siderastreidae	*Siderastrea*	*radians*	19,387	DQ643838

Results

The mitochondrial genome of *D. dianthus*, with a length between 16,311bp and 16,222bp, presented a nucleotide composition with 35% of GC content, similar to those found in other corals (Lin *et al.* 2011; Arrigoni *et al.* 2014). The organization of mitochondria showed the same rearrangement described for *L. pertusa* (Emblem *et al.* 2011): the mitogenome contains 13 protein-coding genes, 2 transfer RNA genes, 2 ribosomal RNA genes, and a group I intron, which interrupts the *nd5* gene including eight protein-coding genes and *rns*,. All protein-coding genes had methionine (ATG) as the translation initiation codons, except for *cob* and *nad2*, which had TAT and TTA respectively, and TAA and TAG as complete stop codons. The two largest non-coding regions were between *nad5* and *cob* genes, the putative control region (Emblem *et al.* 2011), and between *nad6* and *trnW* genes. The former region was responsible for length variation of mitogenome at interspecific as well as intraspecific level: small insertions and deletions (INDELs) that range from 72 bp to 150 bp in length were detected among *L. pertusa* (16,150 bp), the Italian specimen of *D. dianthus* (16,222 bp) and the Chilean one (16,313 bp) (Table 6.4).

Except for the INDELs in the mitochondrial control region, astonishing genetic similarity was found between *L. pertusa* and *D. dianthus*: 99.47% of nucleotides were identical. The entire variability of the mitochondrial genome of the two species, based on the comparison between two specimens of *D. dianthus* and three individuals of *L. pertusa* (excluding the control region), is represented by 87 nucleotide substitutions, of which only 22 were non-synonymous (Table 6.5, Annexe 3).

Table 6.4. Annotation of mitochondrial complete genome od *D. dianthus (Dd)* and *L. pertusa (Lp)* using DOGMA (Wayman *et al.* 2014). CDS=coding sequence

	***Lp*KC875349**		***Lp*KC875348**		***Lp*FR821799**			***Dd*432**			***Dd*636**		***Dd* CDS gene**	
Gene	**start**	**end**	**start**	**end**	**start**	**end**	**Gene**	**start**	**end**	**Gene**	**start**	**end**	**start**	**end**
nad5	1	759	1	759	1	759	*nad5*	1	759	*nad5*	1	759	1	759
nad1	845	1768	845	1768	845	1768	*nad1*	845	1768	*nad1*	953	1762	760	1683
atp6	1824	2519	1824	2519	1824	2519	*atp6*	1824	2066	*atp6*	1821	1931	1684	2379
							atp6	1919	2518	*atp6*	1916	2515		
nad4	2522	3964	2522	3964	2522	3964	*nad4*	2521	3297	*nad4*	2518	3030	2380	3822
										nad4	2900	3961		
rrnS	4876	4919	4876	4919	4876	4919	*rrnS*	4875	4918	*rrnS*	4873	4916		
cox3	5001	5768	5001	5768	5001	5768	*cox3*	5000	5767	*cox3*	4998	5765	3823	4590
cox2	5780	6451	5780	6451	5780	6448	*cox2*	5779	6369	*cox2*	5777	6448	4591	5265
							cox2	6359	6451					
nad4l	6474	6770	6474	6770	6474	6770	*nad4l*	6474	6608	*nad4l*	6471	6605	5266	5565
							nad4l	6587	6769	*nad4l*	6562	6768		
nad3	6787	7116	6787	7116	6787	7116	*nad3*	6786	7115	*nad3*	6785	7114	5566	5895
nad5	7175	8272	7175	8272	7175	8272	*nad5*	7174	8271	*nad5*	7173	8084	5896	6993
cob	9039	10154	9039	10154	9040	10155	*cob*	9218	9475	*cob*	9114	10229	6994	8109
							cob	9471	10334					
nad2	10387	11475	10387	11475	10388	11434	*nad2*	10569	11657	*nad2*	10462	11550	8110	9198
nad6	11504	11992	11504	11992	11505	11993	*nad6*	11686	11973	*nad6*	11579	12067	9199	9687
trnW-uca	12416	12485	12416	12485	12416	12485	*trnW-uca*	12575	12644	*trnW-uca*	12491	12560		
trnS-cga	12588	12640	12588	12640	12589	12641	*trnS-cga*	12747	12799					
cox1	12646	14199	12646	14199	12647	14200	*cox1*	12805	14358	*cox1*	12723	14276	9688	11241
trnM-cau	14207	14277	14207	14277	14208	14278	*trnM-cau*	14366	14436	*trnM-cau*	14284	14354		
rrnL	15385	15643	15385	15643	15386	15644	*rrnL*	15548	15806	*rrnL*	15460	15718		
rrnL	15784	16046	15784	16046	15785	16047	*rrnL*	15947	16209	*rrnL*	15857	16119		

Table 6.5. Pairwise species non-synonymous substitutions with nucleotide (NT) and amino acid (AA) location

				***Dd*432*Lp*KC875348**				***Dd*432*Lp*KC875349**				***Dd*432*Lp*FR821799**				***Dd*432*Dd*636**				***Dd*636*Lp*KC875348**				***Dd*636*Lp*KC875348**				***Dd*636*Lp*FR821799**			
#	**AA**	**NT**	**Gene**	**AA**				**AA**				**AA**				**AA**				**AA**				**AA**				**AA**			
1	26	77	*nad5*	26	R	⟹	K	26	R	⟹	K	26	R	⟹	K					26	R	⟹	K	26	R	⟹	K	26	R	⟹	K
2	38	113	*nad5*	38	T	⟹	I	38	T	⟹	I	38	T	⟹	I	38	T	⟹	I												
3	165	493	*nad5*	165	V	⟹	I	165	V	⟹	I	165	V	⟹	I	165	V	⟹	I												
4	351	1051	*nad1*													351	K	⟹	Q	351	Q	⟹	K	351	Q	⟹	K	351	Q	⟹	K
5	427	1280	*nad1*	427	C	⟹	F	427	C	⟹	F					427	C	⟹	F									427	F	⟹	C
6	1403	4208	*cox3*													1403	N	⟹	S	1403	S	⟹	N	1403	S	⟹	N	1403	S	⟹	N
7	1408	4225	*cox3*	1408	G	⟹	S	1408	G	⟹	S	1408	G	⟹	S	1409	G	⟹	S												
8	1499	4498	*cox3*	1499	S	⟹	G	1499	S	⟹	G	1499	S	⟹	G					1500	S	⟹	G	1500	S	⟹	G	1500	S	⟹	G
9	1685	5056	*cox2*	1685	G	⟹	R	1685	G	⟹	R	1685	G	⟹	R	1686	G	⟹	R												
10	2289	6865	*nad5*									2288	E	⟹	K													2289	E	⟹	K
11	2471	7411	*cob*									2470	T	⟹	P													2471	T	⟹	P
12	2698	8097	*cob*	2698	L	⟹	F	2698	L	⟹	F									2699	L	⟹	F	2699	L	⟹	F				
13	2863	8589	*nad2*													2863	F	⟹	L	2863	L	⟹	F	2863	L	⟹	F	2863	L	⟹	F
14	2930	8789	*nad2*													2930	H	⟹	R	2930	R	⟹	H	2930	R	⟹	H	2930	R	⟹	H
15	3054	9161	*nad2*									3053	L	⟹	S													3054	L	⟹	S
16	3087	9260	*nad6*									3086	L	⟹	P													3087	L	⟹	P
17	3191	9574	*nad6*	3191	Y	⟹	H													3192	Y	⟹	H								
18	3221	9664	*nad6*	3221	L	⟹	I	3221	L	⟹	I	3221	L	⟹	I					3222	L	⟹	I	3222	L	⟹	I	3222	L	⟹	I
19	3348	10046	*cox1*	3348	A	⟹	V	3348	A	⟹	V	3348	A	⟹	V					3349	A	⟹	V	3349	A	⟹	V	3349	A	⟹	V
20	3354	10064	*cox1*	3354	V	⟹	A	3354	V	⟹	A	3354	V	⟹	A					3355	V	⟹	A	3355	V	⟹	A	3355	V	⟹	A
21	3389	10166	*cox1*									3388	S	⟹	F													3389	S	⟹	F
22	3713	11141	*cox1*	3713	R	⟹	K	3713	R	⟹	K	3713	R	⟹	K					3714	R	⟹	K	3714	R	⟹	K				

The values of dN/dS ratio obtained from pairwise comparison between the mitochondrial protein-coding regions of individuals from both species ranged from 0.13 to 0.30. Higher values of substitution ratio, due to mathematic artefacts (as when only one substitution occurs and it is non-synonymous), were found between specimens of *L. pertusa* (Table 6.6).

Table 6.6. Computation of non-synonymous (dN) andsynonymous (dS) substitutions between mitochondrial protein-coding genes of *D. dianthus* (*Dd*) and *L. pertusa* (*Lp*) using one approximate method (NG) and tree maximum-likelihood methods (GY-HKY; MS; MA) (Zhang *et al.* 2006)

Pairwise Sequence	Method	Ka=dN	Ks=dS	Ka/Ks	P-Value(Fisher)	Length	Substitutions	S-Substitutions	N-Substitutions
*Dd*432*Dd*636	NG	0,00105426	0,00738853	0,14269	6,38E-02	11127	28	19	9
	GY-HKY	0,00103991	0,00780277	0,13328	3,46E-03	11127	28	19,0493	8,9507
	MS	0,00120913	0,00911783	0,13261	2,04E-02	11127	28	16,8877	11,1123
	MA	0,00111943	0,00849681	0,13175	2,57E-03	11127	28	18,0081	9,9919
*Dd*432*Lp*KC875348	NG	0,00151378	0,00618744	0,24465	0,000194732	11193	29	16	13
	GY-HKY	0,00147761	0,00678233	0,21786	1,27E-01	11193	29	16,0446	12,9554
	MS	0,00145719	0,00716942	0,20325	5,85E-01	11193	29	16,,0453	12,9547
	MA	0,00149592	0,0071832	0,20825	2,11E+00	11193	29	15,5980	13,4020
*Dd*432*Lp*FR821799	NG	0,00174798	0,00657818	0,26572	0,000305985	11187	32	17	15
	GY-HKY	0,00174679	0,00666052	0,26226	0,000133266	11187	32	17,0509	14,9491
	MS	0,0017217	0,00701057	0,24559	2,37E+00	11187	32	17,0472	14,9528
	MA	0,00180584	0,00717326	0,25175	0,000138073	11187	32	15,9324	16,0676
*Dd*432*Lp*KC875349	NG	0,00139724	0,00618725	0,22583	0,000111185	11193	28	16	12
	GY-HKY	0,00135939	0,00686159	0,19812	5,55E-01	11193	28	16,0428	11,9572
	MS	0,00134202	0,00722641	0,18571	2,64E-01	11193	28	16,0436	11,9564
	MA	0,00138454	0,00726459	0,19059	9,52E-01	11193	28	15,5516	12,4484
*Dd*432*Lp*KC875349	NG	0,00187087	0,00542254	0,34502	0,00136247	11154	30	14	16
	GY-HKY	0,00182133	0,00600644	0,30323	0,000711538	11154	30	14,0360	15,9640
	MS	0,0019595	0,00650537	0,30121	0,00171439	11154	30	11,8204	18,1796
	MA	0,00189377	0,00631826	0,29973	0,00217506	11154	30	12,8315	17,1685
*Dd*636*Lp*KC875348	NG	0,00140188	0,00542062	0,25862	0,00106907	11160	26	14	12
	GY-HKY	0,00134558	0,00632621	0,21270	2,50E+00	11160	26	14,0360	11,9640
	MS	0,00134558	0,00632621	0,21270	2,50E+00	11160	26	14,0360	11,9640
	MA	0,00137335	0,00649319	0,21151	6,87E+00	11160	26	13,6246	12,3754
*Dd*636*Lp*KC875349	NG	0,00128497	0,00542045	0,23706	0,000405305	11160	25	14	11
	GY-HKY	0,00122824	0,0064301	0,19101	1,05E+00	11160	25	14,0343	10,9657
	MS	0,00122824	0,0064301	0,19101	1,05E+00	11160	25	14,0343	10,9657
	MA	0,00124788	0,00657364	0,18983	3,68E-01	11160	25	13,7456	11,2544
*Lp*KC875348*Lp*FR821799	NG	0,00092881	0,000768297	1,20892	0,954969	11223	10	2	8
	GY-HKY	0,00088951	0,000903263	0,98477	0,929985	11223	10	1,9987	8,0013
	MS	0,00088951	0,000903263	0,98477	0,929985	11223	10	1,9987	8,0013
	MA	0,000874244	0,000974582	0,89705	0,605808	11223	10	1,9436	8,0564
*Lp*KC875348*Lp*KC875349	NG	0,000115971	NA	NA	NA	11229	1	NA	1
	GY-HKY	0,000117817	2,36E-06	50,00000	0,367879	11229	1	0,0066	0,9934
	MS	0,000117817	2,36E-06	50,00000	0,367879	11229	1	0,0066	0,9934
	MA	0,000108301	2,17E-06	50,00000	0,367879	11229	1	0,0044	0,9956
*Lp*KC875349*Lp*FR821799	NG	0,000812653	0,000768274	1,05776	0,941351	11223	9	2	7
	GY-HKY	0,000778073	0,000903374	0,86130	0,589175	11223	9	1,9299	7,0701
	MS	0,000778073	0,000903374	0,86130	0,589175	11223	9	1,9299	7,0701
	MA	0,000756522	0,00100953	0,74938	0,272516	11223	9	2,0420	6,9580

Other interesting results were obtained from estimation of uncorrected p-distances among different scleractinian families and genera. The divergence between genera ranged from 4% to 8%, and from 0.2% to 1% between species (Tables 6.7 and 6.8). Genetic distance between *Lophelia* and *Desmophyllum* genera were estimated equal to 0.8%, exactly the same value obtained between two *D. dianthus* individuals.

Table 6.7. Genetic divergences among scleractinian species. ACR= Acroporidae; AGA=Agariciidae; POR=Poritidae; POC= Pocilloporidae. #= number assigned to the species.

#	Acroporidae species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	ACR *Acropora aspera* KF448532	-																		
2	ACR *Acropora digitifera* KF448535	0,000	-																	
3	ACR *Acropora humilis* KF448528	0,000	0,000	-																
4	ACR *Acropora florida* KF448533	0,000	0,000	0,000	-															
5	ACR *Acropora muricata* KF448529	0,000	0,000	0,000	0,000	-														
6	ACR *Acropora hyacinthus* KF448531	0,000	0,000	0,000	0,000	0,000	-													
7	ACR *Acropora divaricata* KF448537	0,001	0,001	0,001	0,001	0,001	0,001	-												
8	ACR *Acropora robusta* KF448538	0,001	0,002	0,001	0,001	0,002	0,001	0,001	-											
9	ACR *Acropora nasuta* KF448536	0,002	0,002	0,002	0,002	0,002	0,002	0,001	0,002	-										
10	ACR *Acropora horrida* KF448530	0,002	0,002	0,002	0,002	0,002	0,002	0,002	0,002	0,002	-									
11	ACR *Acropora tenuis* AF338425	0,005	0,005	0,005	0,005	0,005	0,005	0,005	0,005	0,005	0,006	-								
12	ACR *Acropora yongei* KF448534	0,004	0,005	0,004	0,005	0,004	0,004	0,004	0,004	0,005	0,005	0,001	-							
13	ACR *Isopora palifera* KJ634270	0,025	0,025	0,025	0,023	0,025	0,025	0,025	0,025	0,025	0,025	0,024	0,023	-						
14	ACR *Isopora togianensis* KJ634268	0,024	0,025	0,024	0,023	0,025	0,025	0,025	0,025	0,025	0,025	0,024	0,023	0,002	-					
15	ACR *Anacropora matthai* AY903295	0,060	0,060	0,060	0,060	0,060	0,060	0,059	0,059	0,060	0,060	0,060	0,059	0,059	0,059	-				
16	ACR *Montipora cactus* AY903296	0,061	0,061	0,061	0,061	0,061	0,061	0,061	0,061	0,061	0,061	0,061	0,060	0,060	0,060	0,007	-			
17	ACR *Astreopora explanata* KJ634269	0,062	0,062	0,062	0,062	0,062	0,062	0,062	0,062	0,063	0,063	0,063	0,063	0,063	0,063	0,071	0,073	-		
18	ACR *Astreopora myriophthalma* KJ634272	0,063	0,063	0,063	0,063	0,063	0,063	0,063	0,063	0,063	0,063	0,064	0,063	0,064	0,064	0,072	0,073	0,002		
19	ACR *Alveopora sp.* KJ634271	0,076	0,077	0,076	0,076	0,076	0,076	0,076	0,077	0,076	0,077	0,078	0,077	0,077	0,077	0,065	0,066	0,077	0,077	-

#	Agariciidae species	1	2
1	AGA *Agaricia humilis* DQ643831	-	
2	AGA *Pavona clavus* DQ643836	0,039	-

#	Poritidae species	1	2	3
1	POR *Goniopora columna* JF825141	-		
2	POR *Porites okinawensis* JF825142	0,043	-	
3	POR *Porites panamensis* KJ546638	0,043	0,011	-

#	Pocilloporidae species	1	2	3	4	5	6
1	POC *Pocillopora damicornis* EF526302	-					
2	POC *Pocillopora eydouxi* EF526303	0,001	-				
3	POC *Pocillopora damicornis* EU400213	0,001	0,002	-			
4	POC *Seriatopora caliendrum* EF633601	0,065	0,065	0,065	-		
5	POC *Seriatopora hystrix* EF633600	0,065	0,065	0,065	0,009	-	
6	POC *Stylophora pistillata* EU400214	0,058	0,058	0,058	0,035	0,036	-

Table 6.8. Genetic divergences between *D. dianthus* and *L. pertusa* species. CAR= Caryophylliidae. SML= Santa Maria di Lueca; ADR= Adriatic; IJC= Isla Jaime Chile.

#	***D.dianthus* vs *L.pertusa* Mt complete genome**	**1**	**2**	**3**	**4**	**5**
1	CAR *L. pertusa* FR821799-Norway Nordleska	-				
2	CAR *L. pertusa* KC875348-Norway Korallen	9E-04	-			
3	CAR *L. pertusa* KC875349-Italia SML	9E-04	6E-05	-		
4	CAR *D. dianthus* 636- Italia ADR	0,008	0,008	0,008	-	
5	CAR *D. dianthus* 432-Chile IJC	0,008	0,008	0,008	0,012	-

#	***D.dianthus* vs *L.pertusa* Mt genome without INDEL**	**1**	**2**	**3**	**4**	**5**
1	CAR *L. pertusa* FR821799-Norway Nordleska	-				
2	CAR *L. pertusa* KC875348-Norway Korallen	9E-04	-			
3	CAR *L. pertusa* KC875349-Italia SML	9E-04	6E-05	-		
4	CAR *D. dianthus* 636-Italia ADR	0,008	0,008	0,008	-	
5	CAR *D. dianthus* 432-Chile IJC	0,008	0,008	0,007	0,009	-

Estimations were performed separately for the putative control region, where pairwise comparisons between 15 *D. dianthus* and *L. pertusa* individuals showed genetic distance ranging from 9% to 14% between genera, and from 0.3 to 14% at intraspecific level (Table 6.9).

Table 6.9. Genetic divergence between *D. dianthus* (*Dd*) and *L. pertusa* (*Lp*) individuals using only putative control region sequences

#	**Individuals**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
1	*Lp*FR821799	-													
2	*Lp*KC875348	0,004	-												
3	*Lp*KC875349	0,004	0,000	-											
4	L*p*296	0,002	0,004	0,004	-										
5	*Lp*272	0,002	0,003	0,003	0,000	-									
6	*Dd*ARG472	0,108	0,110	0,110	0,107	0,090	-								
7	*Dd*AUS566	0,103	0,104	0,104	0,100	0,082	0,018	-							
8	*Dd*MNZ601	0,098	0,100	0,100	0,098	0,093	0,132	0,125	-						
9	*Dd*SML62	0,114	0,113	0,113	0,114	0,110	0,115	0,106	0,034	-					
10	*Dd*ADR635	0,101	0,102	0,102	0,102	0,101	0,127	0,123	0,031	0,009	-				
11	*Dd*ADR636	0,102	0,103	0,103	0,103	0,103	0,130	0,126	0,032	0,011	0,003	-			
12	*Dd*IJC433	0,088	0,090	0,090	0,088	0,087	0,104	0,100	0,086	0,103	0,088	0,090	-		
13	*Dd*ILC681	0,091	0,093	0,093	0,091	0,090	0,125	0,120	0,143	0,107	0,147	0,149	0,000	-	
14	*Dd*IJC432	0,096	0,098	0,098	0,098	0,090	0,132	0,128	0,147	0,104	0,144	0,146	0,000	0,002	-

Discussion

Mitochondrial gene order rearrangement and its phylogenetic implications had recently been reported in Scleractinia (Emblem *et al.* 2011; Lin *et al.* 2011; Lin *et al.* 2012; Lin *et al.* 2014). What has not been reported yet was the extremely high level of genetic similarity between the two genera, described so far only as morphologically different (Table 6.10, Figure 6.1)(see Cairns 1994). Moreover, intriguing results were obtained

from the dN/dS ratio where mitochondrial genome of *L. pertusa* and *D. dianthus* appears to be experiencing neutral selection. Since through 16,000 bp more than 99% were identical nucleotides, and just 25% of the all differences were detected as non-synonymous changes, any inferences on positive (adaptation) selection could hardly be interpretable. In the case of *L. pertusa* individuals from Norwegian fjords and Mediterranean Sea, evidence of positive selection was detected, but without any evolutionary relevance (Flot *et al.* 2013). Considering that more sensitive statistical procedure such as the Z test require at lest 10 synonymous and 10 non-synonymous mutations for their assumptions to be met (Nei and Kumar 2000), as well as (Flot *et al.* 2013) pointed out, positive selection was not statistically supported using Fisher's exact test due to the low number of substitutions in protein-coding regions. Therefore values obtained in this study could be attributable to an artefact resulting from the analyses. For instance, in the case of the *L. pertusa* individuals previously mentioned, the adaptive evolution experience was inferred due to the fact that only one substitution (non-synonymous) was detected over more than 16,000 bp. Hence, due to the absence of synonymous substitution, the value of dN/dS ratio resulted to be more than 1, which means positive selection for both *L. pertusa* specimens.

Table 6.10. Morphological description of *D. dianthus* and *L. pertusa* (Cairns 1994)

Morphological characters	***D. dianthus***	***L. pertusa***
Coral type	solitary	colonial
Substrate	firmly attached	forming large dendroid colonies by intratentacular budding
Corallum shape	corallum ceratoid, often flaring at calice (trumpet-shaped), attached through a robust pedicel. Largest North Pacific specimen 60x40 mm in calicualr diameter and 50 mm in height, with a pedicel diameter of 20 mm	corallite ceratoid, often slighlty curved, long and slender: up to 25 mm long and usually 4-6 mm in diameter, but some large corallites up to 11 mm in diameter
Coenosteum	-	dense
Calice	circular, elliptical, or scalloped	circular to slightly elliptical
Theca	unifomly covered with small low granules	covered with low, closely spaced, rounded granules
Costae	rarely expressed, but occasionally C_{1-3} are present in upper half of corallum as thin ridges	usually not present, but occasionally C_{1-2} expressed as low ridges near calice
Septa	hexamerally arranged in 5 to 6 cycles according to the formula: S_{1-2}> S_3>>S_4>S_5>S_6. Fourth cycle (48 septa) attained at a calicular diameter of about 7 mm and fifth cycle at a calicular diameter of about 18 mm; a complete sixth cycle (192 septa) is often present in Japanese specimens.	irregularly arranged in 3 o 4 size classes.
Primary & Secundary Septa	S_{1-2} extremely robust, up to 2 mm thick at thecal edge, and up to 11 mm exsert. S_{1-2} have straight, vertical inner edges that define a deep, narrow fossa, the inner edges of opposing S_{1-2} sometimes almost touching in center of fossa.	Six to 12 primary septa define an equal number of sectors, each sector bisected by a smaller secondary septum. Primary septa fairly highly exsert (up to 1.2 mm), quite thin, and relatively narrow, their straight, vertical inner edges reaching halfway to calice center. Secondary septa about half width of primaries and correspondingly less exsert. Lower, inner edges of all septa slightly sinuous.
Tertiary& Quartary Septa	S_4 much smaller than S_3 (50%-70% width) and least exsert of the septal cycles. S_5 only about half width of S_4, but highly exsert, rising well above S_4 and often becoming incorporated into adjacent S_{1-3} in large coralla.	Tertiaries quite small, but slightly enlarged if flanked by a pair of quaternaries.Lower, inner edges of all septa slightly sinuous.
Septal face	smooth, covered with small, fine, rounded granules	smooth, covered by very small, low, rounded granules
Endothecal dissepiments	sparse endothecal dissepiments in elongate coralla	sparse tabular endothecal dissepiments present low in fossa
Fossa	deep and slender	deep and often curved, such that base cannot be seen
Pali	absent	absent
Columella	usually absent, but may consist of up to 5 slender fascicular or papillose element, usually hidden from view in an intact corallum	usually absent, but occasionally expressed as a small crispate lath
Corallum color	light brown or grey	white

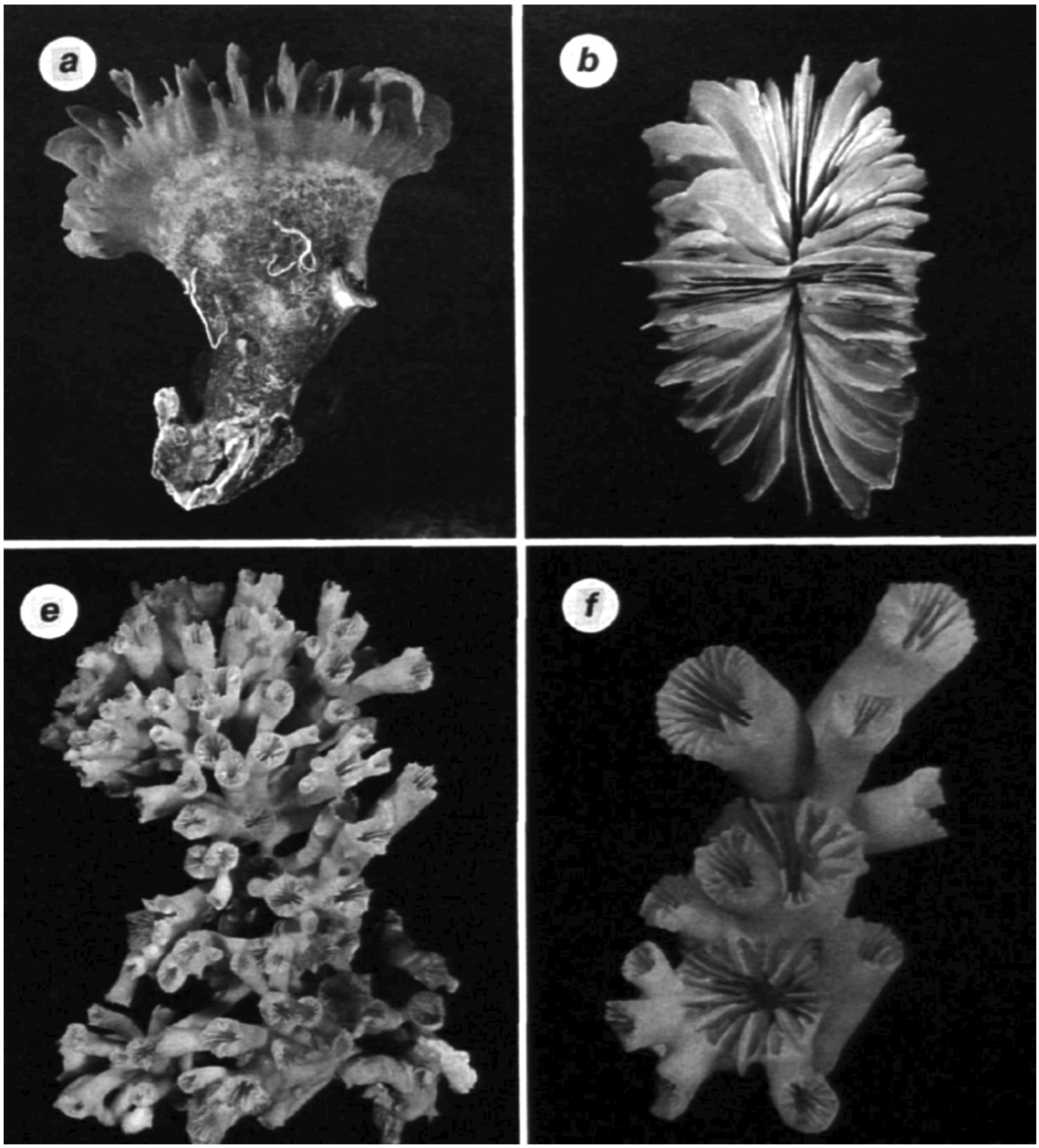

Figure 6.1. Images from Cairns (1994). Specimens of *Desmophyllum dianthus* (a-b, and c-d not shown) and *Lophelia pertusa* (e-f).

Although results from this study were unexpected, genetic similarity and very close phylogenetic relationship between *L. pertusa* and *D. dianthus* have been reported in previous works (Addamo *et al.* 2012; Chapter III, and V), where several molecular markers were used, from different origin (mitochondrial and nuclear), including non-coding and protein-coding genes (exons and introns), and with distinct variation levels (sequences, microsatellites). Regardless of the type of molecular marker considered,

genetic distance performed between both genera always showed a range of values less than 1%, but in many case it was equal to zero. On the other side, genetic divergence between *Desmophyllum* and other caryophyllids genera (*Lophelia* excluded) ranged from 2% to 7% with both non-coding and protein-coding genes, showing a gene-dependent variation correlated to polymorphism level and mutation rate that characterized each marker (Table 6.11). To date, any genetic threshold has been established beyond which scientists could be sure that compared taxa are different species or genera as well as any consensus has been reached in defining a gene as universal DNA barcoding. All these concepts are even more ambiguous in regards to Scleractinia, which is characterized by a very slow evolution rate of mitochondrial and phenomena of extensive interspecific hybridization (van Oppen *et al.* 2001; van Oppen *et al.* 2002); where more than 1500 species of corals showed a wide range of morphological variability and associated genetic incongruences at different phylogenetic level. Furthermore, half of coral species are living in the deep-sea, a more 'stable' habitat than the counterpart tropical shallow water, leading to question if corals from both habitats could been experiencing different evolutionary rate consistent with the habitat where they are living.

Table 6.11. Genetic divergence between *D. dianthus* (*Dd*) and *L. pertusa* (*Lp*), or other *Dd* individual, or *Caryophyllia* species (*C.sp*), or other Hexacorallia species (Outgroup). (Addamo *et al.* 2012; Chapter V)

Markers	***Dd* vs *Lp***	**Dd-Italy vs Dd-Italy**	***Dd* vs *C.sp***	***Dd* vs Outgroup**
16S	0,00	0-0,1	4-7	40
COI	1,10	0,0-1,6	7-8	24
ITS	1,60	0,8-1,1	7	66
28S	0,00	0-0,1	2	19
New Markers	***Dd* vs *Lp***	**Dd-Italy vs Dd-Chile**	***Dd* vs C.sp**	***Dd* vs Outgroup**
COR2-NAD3	0,00	0,00	2,25	0,28
COR3-AMPt1	0,30	0,00	3,00	0,34
COR4-AMPt2	0,00	0,00	3,30	0,30
COR6-SIAH1	0,10	0,00	2,10	0,20
COR7-Actin	0,30	0,00	6,30	0,13
COR10-ßActin	0,30	0,00	7,00	0,15
COR12-Helciase	2,00	0,20	6,00	0,27
COR14-NAD5	0,20	0,00	4,00	0,29
COR15-Creatine kinase	0,00	0,00	3,00	0,21
COR17-NCAH like	0,00	0,00	4,00	0,25
COR21-UBB	0,60	1,60	6,00	0,14
COR24-Heat shock like	0,30	0,00	4,60	0,31
COR25-16S rDNA	0,00	0,00	2,70	0,33
COR26-ATP6NAD4	0,20	0,00	5,00	0,31

Therefore, the hypothesis that *Lophelia* and *Desmophyllum* could be characterized by an extremely slow evolution rate, was tested using hypervariable genetic markers previously isolated (Chapter III). Microsatellite markers are known to be very powerful genetic tools to study population structure, due to their high mutation rate and high levels of polymorphisms. They are usually species-specific, successful cross-amplifications in closely coral related species, due to the conservative flanking region, even though differentiated by species-specific alleles size (Table 11). As demonstrated in a previous work (Chapter III) 30 microsatellites markers developed for *D. dianthus*, not only were successfully genotyped with clear peak profiles in *L. pertusa*, but individuals of *Lophelia* from the Mediterranean Sea and North Atlantic Ocean, also presented the same allele size range of *D. dianthus* (Table 6.12); and even more interesting were the results reporting a lacking of difference in allele size between both genera (Annexe 4).

Table 6.12. Comparison of allele size of *D. dianthus* and *L.pertusa* through 30 microsatellites (Chapter III)

	D. dianthus		***L. pertusa***	
Locus	**min**	**max**	**min**	**max**
B4	246	318	275	284
B9	235	292	256	262
B118	157	301	261	296
C6	161	306	216	230
C102	213	345	243	245
C107	244	259	250	256
L7	202	304	237	241
L13	80	141	89	104
L16	98	161	110	110
L22	119	201	155	168
L24	149	232	162	174
L34B	158	240	167	167
L41	132	210	162	168
L50	189	290	194	266
L56	115	156	128	128
L57	109	173	114	120
L58	105	178	117	121
L73	154	190	158	166
L82	101	149	116	134
L83	224	266	227	242
L84	135	250	160	204
L86	143	158	149	149
L90	90	134	102	104
L91	199	245	214	220
L96	93	156	108	114
L98	129	151	135	141
L100	110	147	122	122
L102	123	185	127	140
L107	110	224	158	164
L109	128	203	137	152

Nevertheless, similar microsatellite electromorphs can arise from independent mutational events and such alleles can be not identical by descent (Estoup *et al.* 1995). Due to this phenomenon, termed size homoplasy, additional comparison analyses were executed using microsatellite sequences: 37 loci, previously characterized for *L. pertusa* (Le Goff and Rogers 2002; Morrison *et al.* 2008) and published in NCBI GenBank, have been used to perform multiple BLAST against genomic DNA libraries from *D. dianthus*, previously obtained from Illumina and 454 platforms (Chapter III, and V)), using TRUFA 0.8.2 (Kornobis Etienne pers. comm.). A compared analysis of electromorph sequences showed that average sequences identity for 1368 separate experiments between *L. pertusa* and *D. dianthus* was about 97 % similar.

The results obtained from this study confirm that the putative mitochondrial control region could be a potential marker for investigating the phylogeography of the genera *Lophelia* (Flot *et al.* 2013) and *Desmophyllum*, but its usefulness in investigating species boundaries it is still uncertain.

It is undoubted that these two genera are significantly more genetically similar than other unambiguous coral genera analysed to date, suggesting that *Desmophyllum* and *Lophelia* should be considered belonging to the same genus or even more, belonging to the same species. But, another intriguing result that is also worth considering is the genetic divergence found at intraspecific level (Chilean specimens vs *Desmophyllum* individuals from other regions) that could reach the same value at genus level (Tables 6.8-6.12), suggesting that *Lophelia* and *Desmophyllum* and Chilean *Desmophyllum* may be three different species within the same genus. Although both taxonomic hypotheses will require to be confirmed, from a integrative systematics point ofview, these results highlighted the high level of taxonomic problems that could be reached in a complex group of organisms, such as Scleractinia, pointing out the importance of defining 'clear taxonomic limits or unquestionable characters' for unambiguous taxonomic assignment.

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DISCUSSION

The main aim of this Thesis was to improve basic scientific knowledge of the evolutionary history of the deep-sea coral *Desmophyllum dianthus*. It is relevant to highlight the enormous lack of information about deep-sea solitary coral species, which constitute pretty much the most of scleractinian deep-sea species identified to date. This unawareness is, in part, due to the presence of several problems when dealing with such corals: solitary species are usually not large in size, hence the common techniques (as epibenthic sledges or rocky dredges) used to sample deep-sea habitats are not adequate to capture these organisms, which often colonize hard substrates. Furthermore, these sampling methodologies are destructive and consequently not adequate to sample the vulnerable ecosystems wherein these species frequently occurs. Moreover, due to their size it is very difficult to collect or record the species using non-destructive methods - such as ROVs or underwater cameras - consequently any kind of study becomes a great challenge. The vast morphological diversity and the lack of adequate diagnostic characters and molecular markers were indeed a barrier when try to deepen the knowledge of these organisms. New approaches and techniques are now providing the essential data to better understand the processes that modulate their evolutionary history. Due to the worldwide distribution of *D. dianthus*, it may be considered a 'species model' for all the rest of solitary deep-sea corals, whose scientific knowledge is completely absent.

Revolutionary Systematics of Scleractinia

The existing classification systems for scleractinians are inadequate and a revised classification system that better reflects new molecular results needs to be adopted as soon as possible (Budd et al. 2010). Although it is clear that *D. dianthus* is phylogenetically more related to certain caryophylliids genera than to others Scleractinia family's genera, the real issue is: to which caryophylliids genera it belongs? Several studies and results from this Thesis have demonstrated the polyphyletic nature of Caryophylliidae (Kerr 2005; Kitahara et al. 2010; Addamo et al. 2012); not only its systematic rearrangement is needed at generic level, but also raising its genera into new families is already taken into consideration (e.g. Deltocyathiidae, Kitahara et al. 2012). Care should be taken while inferring family level phylogenies from one or two species per genus only, because some traditionally recognized genera in Scleractinia are not monophyletic (Benzoni et al. 2007). Since Caryophylliidae is a very large family, and

difficult to collect because it includes azooxanthellate deep-sea species, in addition to them being solitary corals, its rearrangement becomes a higher hurdle than with other families. Despite the clear advances brought by the use of molecular techniques, comprehensive skeletal studies including previously neglected or misidentified macrostructural and microstrucural characters can still provide new information and useful tools for the study of the phylogenetic relationships within the Scleractinia (Benzoni et al. 2007). In fact, in the last few years, several studies have demonstrated their key role in the depth revision of Scleractinia at any phylogenetic level (Budd and Stolarski 2009; Benzoni et al. 2010, 2011, 2012, 2014; Huang et al. 2011; Arrigoni et al. 2012, 2014a, b, c; Budd et al. 2012; Kitahara et al. 2012; Huang et al. 2014a, b).

Nevertheless, the huge effort made to search new informative morphological characters, to improve and apply more sophisticated morphological methodologies, was not equal to the effort in searching new molecular markers that could be more phylogenetically informative than those used to date (such as 16S, COI, and ITS). Although nuclear genes analysis is more difficult that mitochondrial genome analysis - because of the existence of two or more alleles in the individual in contrast to a single haplotype of the mitochondrial region - both mitochondrial and nuclear genes should be applied in phylogenetic analyses. The main reason is that sometimes phylogenetic relationships inferred from mitochondrial gene/regions are different from those derived from nuclear genes, especially if hybridization may occur (van Oppen et al. 2001; Fukami 2008). Difficulty in finding molecular markers suitable for differentiating species-level relationships compounded the problem of large-scale polyphyly. Hence, the need of using at least one good nuclear marker, which is a single-copy gene with a conservative and similar evolutionary tempo through all scleractinian corals but with adequate nucleotide differences among species (Fukami 2008), was already declared. As of today, such request for searching new markers has never been satisfied, leaving the molecular analysis at a stationary point. A clear example is given from *Cladocora caespitosa* (*incertae sedis*) and *Oculina patagonica* (Oculinidae) whose close relationship is already taken into consideration in studies at morphological level, but with some unsatisfactory results at molecular level (F. Benzoni pers. comm.). Studies conducted during this Thesis demonstrated how deeply limited these commonly used markers can be, thus the new molecular markers developed here are presented as

potentially informative tools for phylogenetic studies, such as in the case of *C. caespitosa* and *O. patagonica*, whose close relationship has finally been demonstrated at molecular level. Other examples of the usefulness of the new provided markers was shown in the case of phylogenetic relationship of caryophylliids species *Desmophyllum dianthus, Lophelia pertusa, Caryophyllia smithii, C. calveri*, and *Stephanocyathus regia*, showing that this generic subdivision should be reconsidered. The same suggestion is also argued for the caryophylliids clades of genera *Paraconotrochus, Conotrochus, Odontocyathus* and *Vaughanella.* Results have also confirmed the importance of including more azooxanthellate species in systematics studies of Scleractinia, demonstrating how many questions are still remaining unanswered while many others arise.

Population genetics of *Desmophyllum dianthus*

Population genetics of corals is marginally explored, even further for deep-sea corals due to the cited problems related to sampling methods and habitats. Studies conducted in this Thesis not only have improved knowledge on *D. dianthus* populations' structure, but they have also allowed to infer about its reproductive strategy, larval dispersal and environmental factors that can influence it. Besides being one of the few studies conducted at wide scale for corals, analyses performed in this Thesis demonstrated interesting results on global, broad and large scale, showing unexpected genetic connectivity between areas geographically distant areas - such as Australia and Argentina - where the deep-ocean circulation could play a key role. Own genetic characteristics were also detected for Chilean individuals, the only shallow-water population studied of *D. dianthus*, leading to consider it isolated from others, hence a subpopulation subjected to a possible speciation process. There is no doubt that in-depth research should be conducted at larger scale in order to answer all questions arising from the present study. Moreover, even though microsatellites have been demonstrated to be very informative, other molecular markers such as SNPs (i.e. single nucleotide polymorphism) - which may be more efficient thanks to the large number used in the analysis - should be taken into consideration for population genetics studies at small scale. Information about reproductive strategy (study already in progress, Waller, pers. comm.) could improve the knowledge of the species and the scientific understanding of its larval dispersal.

The stormy species concept in corals: *Desmophyllum dianthus* vs *Lophelia pertusa*

Although the species concept is not an easy issue in general, it seems that when it comes to Scleractinia it is even more difficult to establish boundaries, either at morphological or molecular level. Delimitation and identification of coral species is rendered difficult by intra- and interpopulation morphological variations that are often similar in range to currently recognized species (Veron 1995; Flot et al. 2008) or even greater than the differences in-between them. Although molecular analyses can identify cryptic species, genetic support for unambiguous species-level divergence among hard coral sister taxa is indeed documented, but tends to be the exception rather than the rule (Mackenzie et al. 2004). Moreover, crossings between morphologically different corals, currently considered as distinct species, have been documented to bring out intermediate morphologies, increasing the general confusion (Vollmer and Palumbi 2002).

Since it is not subjected to ecophenotypic variation, the DNA of an organism is commonly used as source of information to assess the intraspecific genetic diversity and phenotypic plasticity respect to the interspecific differences. DNA sequences markers are well suited for such purpose as they generally cross-amplify over a wide range of species, genera and families (Flot et al. 2008). In these cases, characters such as reproductive ecology will be useful to support the genetic data; thus, investigation of ecological and skeletal characters is needed to compare them with the molecular data (Fukami 2008).

There is no doubt that additional analyses (e. g. morphological studies, life history knowledge, behaviour, habitat needs, etc.) should be added to results obtained from this Thesis in order to avoid any speculation, but it is also evident that astonishing genetic similarity between *D. dianthus* and *L. pertusa* lead also to ask if even the first morphological characters in the dichotomous key of Scleractinia (i.e. colonial or solitary, Veron 2000; Cairns and Kitahara 2012) could still be an undisputed morphological character used to identify coral species.

***Desmophyllum dianthus*: the elected scleractinian species**

Desmophyllum dianthus has been defined as one of the simplest coral in morphology (Cairns 1982). Even though this definition could better reflect (just) the number of

morphological characteristics used at taxonomic level, it is clear that 'simple is not synonym of easy'. The extremely high and inexplicable morphological variation of *D. dianthus*, its amazing genetic similarity with *L. pertusa*, its particular distribution and its populations structure, lead to considered *D. dianthus* as one of the 'precious' corals, whose protection should be seriously and urgently taken into consideration.

It is important to stress that bottom trawling together with other fishing methods highly impact the sea floor and the consequences for the deep benthic communities is largely known (Hall-Spencer et al. 2002; Lumsden et al. 2007; Althaus et al. 2009; Orejas et al. 2009; Maynou and Cartes 2012; Norse et al. 2012). Considering the destructive effect of anthropogenic activity in deep-sea coral reefs, the slow growth rates of *D. dianthus* and in general of cold water corals - compared to its tropical counterparts -, it is rightfully suspected that the recovery after impacts of trawling might take a long time in deep waters. It is clear that damages on these communities would have dramatic consequences for the species.

Although studies conducted during this Thesis have improved scientific knowledge on systematics and population genetics of *D. dianthus* and other corals, even more questions have arisen from the obtained results, encouraging the endevours to continuite in achieving ulterior studies related to deep-sea corals research.

CONCLUSIONS

The overall conclusions reached from the results presented in this Thesis are the following:

1. Phylogenetic analyses performed with four molecular markers (mitochondrial 16S and COI genes, and nuclear 28S and ITS fragments) described *Desmophyllum dianthus* as belonging to the 'robust corals', in one of the polyphyletic clades of Caryophylliidae, and that it is closely related to *Lophelia pertusa*.

2. Molecular analyses combined with environmental information have not reported specimens' zonation by geography or by depth currents for *D. dianthus* individuals in the Mediterranean Sea.

3. Nuclear ITS and mitochondrial 16S have not provided genetic structure among individuals from both northern and southern hemispheres. On the contrary, molecular markers showed haplotypes shared between individuals from South Pacific and Mediterranean Sea.

4. Morphological analyses of skeletal and cnidocysts characters have not detected environmental or geographic patterns for the high morphological variability that characterizes *D. dianthus*.

5. The 3D geometric morphometric approach has been applied for the first time in a solitary coral, providing potential informative characters for morphological differentiation at geographical level.

6. Although re-evaluation of previously discarded organic characters of polyps, such as cnidocysts, proved them being potentially informative at intra- and interspecific levels, results suggested that further studies with other corals are needed to confirm their utility.

7. Twenty four microsatellites were developed and characterized for *D. dianthus*. Successful cross amplifications in 46 coral species, representing 40 genera and 10 families, indicated that new molecular tools for other scleractinian, not yet genetically analysed yet, can be accomplished.

8. On a global scale, genetic discontinuity was found between the northern and southern hemisphere populations of *D. dianthus*. However, due to the lack of sampling sites,

probable stepping-stone corridors are not yet excluded, and further studies are still necessary.

9. On large scale, distinct genetic patterns were reported for the northern and southern hemispheres, where deep-ocean circulation may significantly drive the larval dispersal. Genetic connectivity has been found between the Mediterranean Sea and North Atlantic Ocean populations, in particular with the Galician locality (Galician Bank). Instead, peculiar genetic pattern characterized the Cantabrian and Irish populations, which seem to have a low gene exchange with Galician Bank. Gene flow has been detected between samples belonging to Australia and southern Argentina, but those from New Zealand and Chile should be better considered two distinct panmictic populations.

10. Review of phylogenetic signal of molecular markers commonly used in Scleractinia phylogeny showed their limited resolution at different taxonomic levels, suggesting a careful use and interpretation of results arising from them.

11. New developed nuclear molecular markers demonstrated a phylogenetic signal at different levels, bringing in-sight interesting genetic relationships between species that require further studies.

12. The mitochondrial genome of *D. dianthus* presents the same gene rearrangement found, so far, only in *L. pertusa*.

13. Complete mitochondrial genome, microsatellites, protein-coding and non-coding genes revealed that *D. dianthus* and *L. pertusa* are significantly more genetically similar than other coral genera studied to date, suggesting the necessity of a complete and in-depth taxonomic review at generic level.

14. Putative control region of the mitochondrion may be a useful marker to investigate the phylogeography of *D. dianthus* and *L. pertusa* across their worldwide areas of distribution.

15. Genetic data with several molecular markers were obtained for the first time for several coral species, such as *Dendrophyllia laboreli, Paraconotrochus antarctica, Vaughanella* spp., *Stephanocyathus* spp., *Javania* spp. and *Flabellum* spp., for example, providing molecular toolkits for phylogenetic and population genetic studies.

CONCLUSIONES

Las conclusiones generales alcanzadas a partir de los resultados presentados en esta Tesis son las siguientes:

1. Los análisis filogenéticos realizados con cuatro marcadores moleculares (los genes mitocondriales 16S y COI, y los fragmentos nucleares 28S e ITS) identificaron a *Desmophyllum dianthus* como perteneciente al grupo de los corales 'robustos', en uno de los clados de la polifilética familia Caryophylliidae, y estrechamente relacionado con *Lophelia pertusa*.

2. Los análisis moleculares combinados con la información ambiental no han mostrado una zonificación de especímenes de *D. dianthus* por patrón geográfico o por corrientes de profundidad en el mar Mediterráneo.

3. Los genes nucleares ITS y el 16S mitocondrial no han detectado estructura genética alguna entre los individuos de los hemisferios norte y sur. Por el contrario, los marcadores moleculares proporcionaron haplotipos compartidos entre los individuos del Pacífico Sur y del Mediterráneo.

4. Los análisis morfológicos de los caracteres del esqueleto y de los cnidocistos no han detectado patrones ambientales o geográficos claros dentro de la alta variabilidad morfológica que caracteriza a *D. dianthus*.

5. Se ha aplicado por primera vez en un coral solitario un metodología consistente en el análisis a través de morfometría geométrica 3D, proporcionando posibles caracteres informativos para la diferenciación morfológica a nivel geográfico.

6. Aunque la revaluación de caracteres morfológicos de los pólipos, como los cnidocistos, que antes se descartaban, demostró que son potencialmente informativos a nivel intra- e interespecífico, los resultados sugieren que son necesarios más estudios con otros corales para confirmar su utilidad.

7. Se han desarrollado y caracterizado veinticuatro microsatélites en *D. dianthus*. El éxito en las amplificaciones cruzadas en 46 especies de corales, que representan 40 géneros y 10 familias, indicó que las nuevas herramientas moleculares podrían aplicarse a otros escleractinios no analizados aún genéticamente.

8. A escala global, se encontró una cierta discontinuidad genética entre el hemisferio norte y el sur, para las poblaciones de *D. dianthus* analizadas. Sin embargo, debido a la falta de muestreo en ciertas zonas, no puede excluirse la posibilidad de que existan corredores 'stepping-stone', por lo que serán necesarios estudios posteriores.

9. A gran escala, se han hallado distintos patrones genéticos en los hemisferios norte y sur, donde las corrientes profundas pueden influir significativamente en la dispersión de las larvas. Se ha encontrado una conectividad genética entre las poblaciones del mar Mediterráneo y del norte del océano Atlántico, en particular con el banco de Galicia. Por el contrario, resulta interesante que las poblaciones cantábricas e irlandesas tengan un bajo flujo génico con el banco de Galicia. Sí se ha detectado un flujo de genes entre las muestras pertenecientes a Australia y el sur de Argentina, mientras que las de Nueva Zelanda y Chile se deben considerar como procedentes de dos poblaciones panmícticas distintas.

10. La revisión de la información contenida en los marcadores moleculares utilizados en la filogenia del orden Scleractinia mostró su limitada resolución en diferentes niveles taxonómicos, lo que hace que se recomiende un uso y una interpretación cuidadosa de los resultados derivados de esos marcadores.

11. Los nuevos marcadores moleculares de origen nuclear desarrollados mostraron una señal filogenética a diferentes niveles, poniendo en evidencia que hay que profundizar necesariamente en ciertas relaciones filogenéticas entre especies que mostraron patrones inesperados.

12. El genoma mitocondrial de *Desmophyllum dianthus* presenta la misma reorganización génica encontrada, hasta ahora, sólo en *Lophelia pertusa*.

13. El genoma mitocondrial completo, los microsatélites, los genes codificantes para proteínas y los genes no codificantes revelaron que *D. dianthus* y *L. pertusa* son sustancialmente más similares genéticamente que otros géneros de corales estudiados hasta la fecha, lo que sugiere la necesidad de una revisión taxonómica completa y en profundidad a nivel de género.

14. La supuesta región control mitocondrial puede ser un marcador útil para investigar la filogeografía de *D. dianthus* y *L. pertusa* a lo largo de toda su área de distribución.

15. Se han obtenido los primeros datos genéticos, con varios marcadores moleculares, para diferentes corales, tales como *Dendrophyllia laboreli, Paraconotrochus antarctica, Vaughanella* spp., *Stephanocyathus* spp., *Javania* spp. y *Flabellum* spp., por ejemplo, proporcionando un conjunto de herramientas moleculares para estudios filogenéticos y de genética de poblaciones.

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ANNEXES

Annexe 1. List of scleractinian taxa used in the study of Chapter I, with GenBank Accession Numbers. *Shared haplotype with South Pacific population. Samples sequenced by the authors are indicated in bold. Ind ID= identification code for each specimens.

Family	Species	Ind ID	GenBank 16S	GenBank COI	GenBank ITS	GenBank 28S	Haplotype 16S (n° tot ind)	Genotype ITS (n° tot ind)	Adriatic Sea	Ionian Sea	Strait of Sicily
Acroporidae	*Acropora tenuis*		AF338425	AF338425							
Agariciidae	*Agaricia humilis*		DQ643831	DQ643831							
Astrocoeniidae	*Stephanocoenia michelinii*		AF265581	AY451343	AB441413	EU262865					
Caryophylliidae	*Caryophyllia ambrosia*		FJ788113								
Caryophylliidae	*Caryophyllia atlantica*		AF550362	HM018613							
Caryophylliidae	***Caryophyllia calveri***	**A**	**JQ611347**	**JQ611391**	**JQ611298**	**JQ611430**					
Caryophylliidae	***Caryophyllia calveri***	**B**	**JQ611348**	**JQ611392**	**JQ611299**	**JQ611431**					
Caryophylliidae	*Caryophyllia diomedeae*		FJ788116	HM018614							
Caryophylliidae	*Caryophyllia grandis*		FJ788118								
Caryophylliidae	*Caryophyllia grayi*		FJ788119	HM018615							
Caryophylliidae	*Caryophyllia inornata*		AF265599								
Caryophylliidae	*Caryophyllia lamellifera*		FJ788120	HM018616							
Caryophylliidae	*Caryophyllia planilamellata*		FJ788122								
Caryophylliidae	*Caryophyllia ralphae*			HM018617							
Caryophylliidae	*Caryophyllia rugosa*		FJ788123	HM018618							
Caryophylliidae	*Caryophyllia scobinosa*		FJ788124								
Caryophylliidae	***Caryophyllia smithii***	**B**	**JQ611350**	**JQ611394**	**JQ611301**	**JQ611433**					
Caryophylliidae	***Caryophyllia smithii***	**D**	**JQ611349**	**JQ611393**	**JQ611300**	**JQ611432**					
Caryophylliidae	*Caryophyllia transversalis*		FJ788126								
Caryophylliidae	*Caryophyllia unicristata*		FJ788129								
Caryophylliidae	*Ceratotrochus magnaghii*		AF265597								
Caryophylliidae	*Cladocora arbuscula*			AB117292							
Caryophylliidae	*Cladocora caespitosa*		AF265612								
Caryophylliidae	***Cladocora caespitosa***		**JQ611352**	**JQ611396**	**JQ611303**	**JQ611435**					
Caryophylliidae	*Conotrochus funicolumna*			HM018621							
Caryophylliidae	*Crispatotrochus rugosus*		AF265600								
Caryophylliidae	*Dactylotrochus cervicornis*			HM018624							
Caryophylliidae	*Dasmosmilia cf. Lymani*			HM018625							
Caryophylliidae	*Dasmosmilia lymani*		FJ788130								
Caryophylliidae	*Deltocyathus inusitatus*			HM018626							
Caryophylliidae	*Deltocyathus ornatus*			HM018628							
Caryophylliidae	*Deltocyathus rotulus*			HM018629							
Caryophylliidae	*Deltocyathus sarsi*			HM018630							
Caryophylliidae	*Deltocyathus suluensis*			HM018631							
Caryophylliidae	***Desmophyllum dianthus***	**DdBAR 124**	**JQ611339**	**JQ611383**	**JQ611290**	**JQ611422**					
Caryophylliidae	***Desmophyllum dianthus***	**DdDAU 113**	**JQ611327**	**JQ611371**	**JQ611278**	**JQ611410**					
Caryophylliidae	***Desmophyllum dianthus***	**DdGEL 266**	**JQ611340**	**JQ611384**	**JQ611291**	**JQ611423**					
Caryophylliidae	***Desmophyllum dianthus***	**DdGON 115**	**JQ611324**	**JQ611368**	**JQ611275**	**JQ611407**					
Caryophylliidae	***Desmophyllum dianthus***	**DdLIN 130**	**JQ611333**	**JQ611377**	**JQ611284**	**JQ611416**					
Caryophylliidae	***Desmophyllum dianthus***	**DdMAL 136**	**JQ611325**	**JQ611369**	**JQ611276**	**JQ611408**					
Caryophylliidae	***Desmophyllum dianthus***	**DdMAL 137**	**JQ611334**	**JQ611378**	**JQ611285**	**JQ611417**					
Caryophylliidae	***Desmophyllum dianthus***	**DdMAL 193**	**JQ611318**	**JQ611362**	**JQ611269**	**JQ611401**					
Caryophylliidae	***Desmophyllum dianthus***	**DdROC 146**	**JQ611335**	**JQ611379**	**JQ611286**	**JQ611418**					
Caryophylliidae	***Desmophyllum dianthus***	**DdROC 147**	**JQ611336**	**JQ611380**	**JQ611287**	**JQ611419**					
Caryophylliidae	***Desmophyllum dianthus***	**DdSML 09**	**JQ611343**	**JQ611387**	**JQ611294**	**JQ611426**					
Caryophylliidae	***Desmophyllum dianthus***	**DdSML 19**	**JQ611319**	**JQ611363**	**JQ611270**	**JQ611402**					
Caryophylliidae	***Desmophyllum dianthus***	**DdSML 41**	**JQ611337**	**JQ611381**	**JQ611288**	**JQ611420**					
Caryophylliidae	***Desmophyllum dianthus***	**DdSML 57**	**JQ611328**	**JQ611372**	**JQ611279**	**JQ611411**					

Family	Species	Ind ID	GenBank 16S	GenBank COI	GenBank ITS	GenBank 28S	Haplotype 16S (n° tot ind)	Genotype ITS (n° tot ind)	Adriatic Sea	Ionian Sea	Strait of Sicily
Caryophylliidae	***Desmophyllum dianthus***	**DdSML 62**	**JQ611321**	**JQ611365**	**JQ611272**	**JQ611404**					
Caryophylliidae	***Desmophyllum dianthus***	**DdSML 82**	**JQ611320**	**JQ611364**	**JQ611271**	**JQ611403**					
Caryophylliidae	***Desmophyllum dianthus***	**DdSML 86**	**JQ611322**	**JQ611366**	**JQ611273**	**JQ611405**					
Caryophylliidae	***Desmophyllum dianthus***	**DdURA 101**	**JQ611326**	**JQ611370**	**JQ611277**	**JQ611409**					
Caryophylliidae	***Desmophyllum dianthus***	**DdURA 103**	**JQ611330**	**JQ611374**	**JQ611281**	**JQ611413**					
Caryophylliidae	***Desmophyllum dianthus***	**DdURA 127**	**JQ611344**	**JQ611388**	**JQ611295**	**JQ611427**					
Caryophylliidae	***Desmophyllum dianthus***	**DdBAR 110**	**JQ611338**	**JQ611382**	**JQ611289**	**JQ611421**					
Caryophylliidae	***Desmophyllum dianthus***	**DdSML 01**	**JQ611329**	**JQ611373**	**JQ611280**	**JQ611412**	H5 (1)			1	
Caryophylliidae	***Desmophyllum dianthus***	**DdLIN 131**	**JQ611341**	**JQ611385**	**JQ611292**	**JQ611424**		G3 (1)			1
Caryophylliidae	***Desmophyllum dianthus***	**DdBAR 120**	**JQ611323**	**JQ611367**	**JQ611274**	**JQ611406**		G6 (1)	1		
Caryophylliidae	***Desmophyllum dianthus***	**DdSML 26**	**JQ611332**	**JQ611376**	**JQ611283**	**JQ611415**		G7 (2)		1	1
Caryophylliidae	***Desmophyllum dianthus***	**DdSML 32**	**JQ611331**	**JQ611375**	**JQ611282**	**JQ611414**		G9 (1)		1	
Caryophylliidae	***Desmophyllum dianthus***	**DdGAL 85**	**JQ611342**	**JQ611386**	**JQ611293**	**JQ611425**		G14 (4)		4	
Caryophylliidae	***Desmophyllum dianthus***	**DdSML 65**	**JQ611357**				H1 (1)			1	
Caryophylliidae	***Desmophyllum dianthus***	**DdSML 189**	**JQ611358**				H2 (1)			1	
Caryophylliidae	***Desmophyllum dianthus***	**DdBAR 121**	**JQ611359**				H3 (1)		1		
Caryophylliidae	***Desmophyllum dianthus***	**DdROC 148**	**JQ611360**				H4 (1)				
Caryophylliidae	***Desmophyllum dianthus***	**DdGEL 104**	**JQ611361**				H6 (33)*		6	17	10
Caryophylliidae	***Desmophyllum dianthus***	**DdMAL 220**			**JQ611307**			G1 (1)			1
Caryophylliidae	***Desmophyllum dianthus***	**DdMAL 211**			**JQ611308**			G2 (1)			1
Caryophylliidae	***Desmophyllum dianthus***	**DdMAL 195**			**JQ611309**			G4 (1)			1
Caryophylliidae	***Desmophyllum dianthus***	**DdROC 150**			**JQ611310**			G5 (1)		1	
Caryophylliidae	***Desmophyllum dianthus***	**DdGAL 79**			**JQ611311**			G8 (1)		1	
Caryophylliidae	***Desmophyllum dianthus***	**DdSML 34**			**JQ611312**			G10 (1)		1	
Caryophylliidae	***Desmophyllum dianthus***	**DdSML 40**			**JQ611313**			G11 (1)		1	
Caryophylliidae	***Desmophyllum dianthus***	**DdSML 53**			**JQ611314**			G12 (1)		1	
Caryophylliidae	***Desmophyllum dianthus***	**DdSML 63**			**JQ611315**			G13 (1)		1	
Caryophylliidae	***Desmophyllum dianthus***	**DdBAR 50**			**JQ611316**			G15 (1)	1		
Caryophylliidae	***Desmophyllum dianthus***	**DdGEL 267**			**JQ611317**			G16 (8)*	3	3	2
Caryophylliidae	*Lophelia pertusa*		AF550367								
Caryophylliidae	***Lophelia pertusa***		**JQ611345**	**JQ611389**	**JQ611296**	**JQ611428**					
Caryophylliidae	*Odontocyathus weberianus*		AF265594								
Caryophylliidae	*Paracyathus pulchellus*		AF265603								
Caryophylliidae	*Phyllangia mouchezii*		AF265605								
Caryophylliidae	*Polycyathus muellerae*		AF265606								
Caryophylliidae	***Pourtalosmilia anthophyllites***		**JQ611346**	**JQ611390**	**JQ611297**	**JQ611429**					
Caryophylliidae	*Rhizosmilia maculata*		AF265602								
Caryophylliidae	*Rhizosmilia robusta*			HM018664							
Caryophylliidae	*Solenosmilia variabilis*		HM015349								
Caryophylliidae	*Stephanocyathus platypus*		HM015352								
Caryophylliidae	*Stephanocyathus spiniger*		HM015359	HM018665							
Caryophylliidae	*Tethocyathus virgatus*		FJ788131								
Caryophylliidae	*Thalamophyllia gasti*		AF265590								
Caryophylliidae	*Trochocyathus efateensis*			HM018667							
Caryophylliidae	*Trochocyathus rhombocolumna*			HM018668							
Caryophylliidae	*Vaughanella sp.*		AF265595								
Dendrophyliidae	*Tubastraea coccinea*		L76022	DQ445806	AF180110	EU262864					
Euphyllidae	*Catalaphyllia jardinei*		L76000								
Euphyllidae	*Euphyllia ancora*		AF265598	AB441204							
Euphyllidae	*Euphyllia divisa*			AB441203							

Family	Species	Ind ID	GenBank 16S	GenBank COI	GenBank ITS	GenBank 28S	Haplotype 16S (n° tot ind)	Genotype ITS (n° tot ind)	Adriatic Sea	Ionian Sea	Strait of Sicily
Euphyllidae	*Euphyllia glabrescens*			AB441206							
Euphyllidae	*Plerogyra sp.*			HM018663							
Faviidae	*Montastrea faveolata*		AP008977	AP008977	AB065363	AY064532					
Flabellidae	*Flabellum deludens*		AB510170	HM018638							
Flabellidae	***Javania cailleti***		**JO611355**	**JO611399**	**JO611306**	**JO611438**					
Fungiidae	*Fungia(Lobactis) scutaria*		L76005	EU149882							
Meandrinidae	*Dichocoenia stokesi*		AF265607	AB117298							
Merulinidae	*Merulina scabricula*		L76014	AB117284							
Mussidae	*Mussa angulosa*		DQ643834	DQ643834	AB441402	AF549236					
Oculinidae	*Madrepora oculata*		HM015351	HM018659							
Oculinidae	***Madrepora oculata***		**JO611351**	**JO611395**	**JO611302**	**JO611434**					
Pectiniidae	*Pectinia alcicornis*		L76017	AB117385							
Pocilloporidae	*Madracis mirabilis*		EU400212	EU400212	AB441412	EU262845					
Pocilloporidae	***Madracis pharensis***			**JO611400**							
Portiidae	*Porites compressa*		L76020	FJ423971	FJ416592	EU262814					
Siderastreidae	*Siderastrea radians*		DQ643838	DQ643838	AY322609	EU262861					

Annexe 2. List of Scleractinia species included in the analyses of Chapter V. Fam.code= code for the scleractinian families included in the analyses; Gen.code= code for the scleractinian genera included in the analyses; *Species subjected to taxonomic rearrangement.

Family	Genus	*Genus*	*Species*	Fam. code	Gen. code	*	Genes	No. GenBank Access
Acroporidae	*Acropora*	*Acropora*	*austera*	ACR	acropo		CYTB	FJ391989
		Acropora	*cerealis*	ACR	acropo		CYTB	AF099652,FJ391990
		Acropora	*cervicornis*	ACR	acropo		CYTB;12S,COI	AF099654,AY451340,EF597094,KF579899,KF579900,KJ573647,KJ573648
		Acropora	*corymbosa*	ACR	acropo		CYTB	FJ391997
		Acropora	*cytherea*	ACR	acropo		CYTB;12S;16S	AF333054,FJ391995,L75995
		Acropora	*danai*	ACR	acropo		16S	AF550358
		Acropora	*digitifera*	ACR	acropo		CYTB;12S;16S;COI	AB033184,AF099650,AF177043,AF333051,KF448535
		Acropora	*divaricata*	ACR	acropo		COI	KF448537
		Acropora	*donei*	ACR	acropo		CYTB	AB033180
		Acropora	*echinata*	ACR	acropo		CYTB	FJ391985
		Acropora	*florida*	ACR	acropo		CYTB;12S;16S;COI	AB033182,KF448533
		Acropora	*formosa*	ACR	acropo		CYTB;12S,COI	AF099651,AF177042,DQ320494,JQ920462
		Acropora	*gemmifera*	ACR	acropo		CYTB	AB033183,FJ391980,FJ391981,FJ391982
		Acropora	*hemprichii*	ACR	acropo		16S	AF550359
		Acropora	*horrida*	ACR	acropo		CYTB;12S;16S;COI	KF448530
		Acropora	*humilis*	ACR	acropo		CYTB;12S;16S;COI	AF550360,DQ320495,EF363315,EF363316,FJ391978,FJ391979,KF448528,L75996
		Acropora	*hyacinthus*	ACR	acropo		CYTB;12S;16S;COI	AF333053,FJ391988,KF448531
		Acropora	*latistella*	ACR	acropo		CYTB	AF099656,FJ391993
		Acropora	*microphthalma*	ACR	acropo		CYTB	FJ391986
		Acropora	*millepora*	ACR	acropo		CYTB	AF099653,FJ391984
		Acropora	*muricata*	ACR	acropo		CYTB;12S;16S;COI	AF333052,AF550361,FJ391992,KF448529
		Acropora	*nasuta*	ACR	acropo		CYTB;12S;16S;COI	AB033185,KF448536
		Acropora	*palmata*	ACR	acropo		CYTB;COI;12S	AB441246,AB441331,AF099655,AY451341,EF597092,KF579901
		Acropora	*robusta*	ACR	acropo		CYTB;12S;16S;COI	JQ347814,JQ347837,KF448538
		Acropora	*samoensis*	ACR	acropo		CYTB	FJ391994
		Acropora	*sp*	ACR	acropo		16S;12S	JQ347815,JQ347838
		Acropora	*surculosa*	ACR	acropo		12S	EF597093
		Acropora	*tenuis*	ACR	acropo		CYTB;12S;16S;COI	AB033181,AF152244,AF338425,FJ391991,KJ573649,KJ573650
		Acropora	*valida*	ACR	acropo		CYTB	AF099658,FJ391983
		Acropora	*yongei*	ACR	acropo		CYTB;12S;16S;COI	KF448534
		Acropora	*aspera*	ACR	acropo		CYTB;12S;16S;COI	FJ391987,KF448532
	Alveopora	*Alveopora*	*excelsa*	ACR	alveop		COI	AB907084,AB907085
		Alveopora	*japonica*	ACR	alveop		CYTB;COI	AB907089,KJ573651,KJ573652
		Alveopora	*sp*	ACR	alveop		CYTB;12S;16S;COI	AB441245,AB441330,AB907090,AB907091,AF265592,EF597088,KJ634271
		Alveopora	*spongiosa*	ACR	alveop		COI	AB907092,AB907093,AB907094
		Alveopora	*verrilliana*	ACR	alveop		COI	AB907097
	Anacropora	*Anacropora*	*forbesi*	ACR	anacro		CYTB;COI	AB441251,AB441336
		Anacropora	*matthai*	ACR	anacro		CYTB;12S;16S;COI	AB441250
		Anacropora	*matthai*	ACR	anacro		CYTB	AB441335,AY903295
		Anacropora	*sp*	ACR	anacro		CYTB;12S;16S;18S	AB033176,AB033177,AF333046,AY722747,L75992
	Astreopora	*Astreopora*	*explanata*	ACR	astreo		CYTB;12S;16S;COI	AB441254,AB441339,KJ634269
		Astreopora	*listeri*	ACR	astreo		18S	AY722742
		Astreopora	*myriophthalma*	ACR	astreo		CYTB;12S;16S;COI	AB033171,AB441253,AB441338,AF177046,FJ392006,KJ573653,KJ573654,KJ634272
		Astreopora	*sp*	ACR	astreo		COI;12S;16S;18S	AB907076,AB907077,AB907078,AF265591,AY722748,EF597095
	Isopora	*Acropora*	*brueggemanni*	ACR	isopor	*	12S	AF333048
		Acropora	*cuneata*	ACR	isopor	*	12S	AF333049
		Acropora	*nobilis*	ACR	isopor	*	CYTB;12S	DQ320496,FJ391996
		Acropora	*palifera*	ACR	isopor	*	12S;16S	AF177044,AF265593,AF333047
		Acropora	*togianensis*	ACR	isopor	*	CYTB;12S	AF310137,AF333050
		Isopora	*brueggemanni*	ACR	isopor		CYTB;COI	AB033178,AB441247,AB441332
		Isopora	*palifera*	ACR	isopor		CYTB;COI;12S;16S	AB033179,AB441248,AB441333,EF597091,FJ391998,KJ634270
		Isopora	*togianensis*	ACR	isopor		CYTB;COI;12S;16S	AB441249,AB441334,KJ634268
	Montipora	*Montipora*	*aequituberculata*	ACR	montip		CYTB;12S;18S	AB033172,AF333045,AY722772,FJ391999

Family	Genus	*Genus*	*Species*	Fam. code	Gen. code	*	Genes	No. GenBank Access
		Montipora	*altasepta*	ACR	montip		CYTB	AB033175
		Montipora	*angulata*	ACR	montip		18S	AY722773
		Montipora	*aquituberculata*	ACR	montip		CYTB	AF099659
		Montipora	*cactus*	ACR	montip		CYTB;COI;12S;16S	AB441252,AB441337,AY9032962
		Montipora	*capitata*	ACR	montip		CYTB;COI;16S	HQ246516,HQ246610,HQ246611,HQ246612,HQ246613,HQ246706,HQ246707,HQ246708,HQ246709,L7601
		Montipora	*cf*	ACR	montip		CYTB;COI;16S;18S	HQ246493,HQ246494,HQ246495,HQ246510,HQ246511,HQ246512,HQ246607,HQ246608,HQ246609,HQ246614,HQ246615,HQ246703,HQ246704,HQ246705,HQ246710,HQ246711,HQ246712
		Montipora	*circumvallata*	ACR	montip		16S	AF550368
		Montipora	*digitata*	ACR	montip		CYTB;12S;16S	AB033173,AF177045,FJ392000,FJ392001,FJ392002,FJ392004,L75993
		Montipora	*dilatata*	ACR	montip		CYTB;COI;16S	HQ246507,HQ246508,HQ246604,HQ246605,HQ246606,HQ246701,HQ246702
		Montipora	*efforescens*	ACR	montip		CYTB	AB033174
		Montipora	*flabellata*	ACR	montip		CYTB;COI;16S	HQ246505,HQ246506,HQ246601,HQ246602,HQ246603,HQ246698,HQ246699,HQ246700
		Montipora	*foliosa*	ACR	montip		CYTB;COI	FJ392003,JQ920455
		Montipora	*grisea*	ACR	montip		CYTB	KJ573686,KJ573687
		Montipora	*hispida*	ACR	montip		CYTB	FJ392005
		Montipora	*molis*	ACR	montip		18S	AY722776
		Montipora	*patula*	ACR	montip		COI;16S;18S	HQ246464,HQ246465,HQ246467,HQ246468,HQ246469,HQ246470,HQ246594,HQ246595,HQ246596,HQ246597,HQ246690,HQ246691,HQ246692,HQ246693
		Montipora	*peltiformis*	ACR	montip		18S	AY722777
		Montipora	*sp*	ACR	montip		COI;12S;16S	AB907075,JQ347816,JQ347817,JQ347839,JQ347840,JQ920454
		Montipora	*taiwanensis*	ACR	montip		18S	AY722778
		Montipora	*tuberculosa*	ACR	montip		18S	AY722779
		Montipora	*venosa*	ACR	montip		18S	AY722780
		Montipora	*verrilli*	ACR	montip		COI;16S;18S	HQ246454,HQ246455,HQ246456,HQ246457,HQ246458,HQ246598,HQ246599,HQ246600,HQ246694,HQ246695.HQ246696.HQ246697
		Montipora	*verrucosa*	ACR	montip		12S	EF597090
Agariciidae	*Agaricia*	*Agaricia*	*agaricites*	AGA	agaric		COI;12S;28S	AF112120,AF112121,AY451366,AY451367,EF597079,EF597080,EU262791,EU262859
		Agaricia	*fragilis*	AGA	agaric		COI;12S	AY451368,EF597077
		Agaricia	*grahamae*	AGA	agaric		12S;28S	EF597078,EU262823
		Agaricia	*humilis*	AGA	agaric		CYTB;12S;16S;COI;28S	AB441219,AB441304,DQ643831,EF597082,EU262785
		Agaricia	*lamarcki*	AGA	agaric		COI;12S;28S	AY451369,EF597076,EU262779
		Agaricia	*tenuifolia*	AGA	agaric		12S;28S	EF597081,EU262827
		Agaricia	*undata*	AGA	agaric		12S;28S	AF549214,EF597075,EU262789
	Dactylotrochus	*Dactylotrochus*	*cervicornis*	AGA	dactyl		COI;16S;28S	HM018624,HQ439630,HQ439697
	Gardineroseris	*Gardineroseris*	*planulata*	AGA	gardis		CYTB;COI;ITS;12S;18S;28S	AB441218,AB441303,AB441409,EF597084,EU262776
	Leptoseris	*Helioseris*	*cucullata*	AGA	leptos	*	CYTB;COI	AB441220,AB441221,AB441305,AB441306
		Leptoseris	*foliosa*	AGA	leptos		COI;ITS	HE978501,HE978502,HE978506,HE978507
		Leptoseris	*incrustans*	AGA	leptos	*	16S	L76012
		Leptoseris	*yabei*	AGA	leptos		18S;ITS	AB441410
	undefined genus	*Leptoseris*	*sp*	AGA	lptsxx	*	COI;12S;28S	AF549215,AY451373,EF597089,EU262863
	Pachyseris	*Pachyseris*	*sp*	AGA	pachys		12S;28S	EF597085,EU262835
		Pachyseris	*speciosa*	AGA	pachys		CYTB;COI	AB441222,AB441307
	Pavona	*Pavona*	*cactus*	AGA	pavona		CYTB;COI,ITS;12S;16S;18S	AB217876,AB217877,AB217878,AB217879,AB217880,AB217881,AB217882,AB217883,AB217884,AB217885,AB217886,AB217887,AB217888,AB441216,AB441217,AB441301,AB441302,AB441408,AB441408,AF550370,EU233631,EU233632,EU233633,EU233634,EU233635,EU233637,EU233638,EU233641
		Pavona	*clavus*	AGA	pavona		CYTB;COI;12S;16S	DQ643836
		Pavona	*decussata*	AGA	pavona		ITS;28S	AB217889,AB217890,AB217891,AB217892,AB217893,AB217894,AB217895,AB217896,AB217897,AB217898,AB217899,AB217900,AB217901,AB217902,AB217903,AB217904,AB217905,AB217906,AB217907,AB217908.AB217909.AB217910.AB217911.AB217912.AB217913.EU233636.JO966154
		Pavona	*frondifera*	AGA	pavona		COI;12S;16S	AF333055,JQ347825,JQ347847,JQ920448
		Pavona	*varians*	AGA	pavona		12S;16S;28S	AF549254,EF597083,EU262847,L76016
Anthemiphyllidae	*Anthemiphyllia*	*Anthemiphyllia*	*dentata*	ANT	anthem		COI;16S;28S	HM018603,HQ439611,HQ439686
		Anthemiphyllia	*patera*	ANT	anthem		COI;16S;28S	HM018604,HQ439609,HQ439684

Family	Genus	*Genus*	*Species*	Fam. code	Gen. code	*	Genes	No. GenBank Access
		Anthemiphyllia	*spinifera*	ANT	anthem		28S;12S;16S	AF265596,EF597038,EU262852,HQ439610
Astrocoenidae	*Madracis*	*Madracis*	*asanoi*	AST	madrac		COI	HM018656
		Madracis	*carmabi*	AST	madrac		12S;28S	EF596980,EU262844
		Madracis	*decactis*	AST	madrac		CYTB;12S;28S	EF596982,EU262818,EU262873,KJ573678,KJ573679
		Madracis	*formosa*	AST	madrac		12S;28S	AF549243,EF596981,EU262792
		Madracis	*mirabilis*	AST	madrac	*	CYTB;COI;ITS;12S;16S;28S	AF251847,AF251850,AF251852,AF251854,AF251855,AF251856,AF251857,AY451344,EU262806,EU262845 .EU400212
		Madracis	*myriaster*	AST	madrac		CYTB;COI;ITS;16S;18S	AB441226,AB441227,AB441311,AB441312,KJ482946
		Madracis	*pharensis*	AST	madrac		COI;ITS;12S;28S	AF251919,AF251921,AF251935,AF549242,EF596983,EF596984,EU262840,JQ611400
		Madracis	*senaria*	AST	madrac		ITS;12S;28S	AF251904,AF251905,AF251906,AF251907,AF251908,AF251909,AF251910,AF251911,AF251912,AF251913, AF251915.EF596979.EU262837
		Madracis	*sp*	AST	madrac	*	COI	HM018657,HM018658
	Stephanocoenia	*Stephanocoenia*	*michelinii*	AST	stephc	*	CYTB;COI;ITS;12S;16S;28S	AB441228,AB441229,AB441313,AB441314,AB441413,AF265581,AY451343,EF597073,EU262805
	Stylocoeniella	*Stylocoeniella*	*guentheri*	AST	styloc		CYTB;COI;ITS;18S	AB441225,AB441310,AY722791,AY722792,AY722793
Caryophylliidae	*Caryophyllia (Caryophyllia)*	*Caryophyllia*	*grayi*	CAR	caraca		COI;28S	HM018615,HQ439615
	undefined genus	*Caryophylliidae*	*gen*	CAR	carxxx		COI;28S	HM018649,HQ439627,HQ439628,HM018620,HQ439625,HQ439626
	Caryophyllia	*Caryophyllia*	*atlantica*	CAR	caryop		COI;28S	HM018613,HQ439612
		Caryophyllia	*calveri*	CAR	caryop		ITS;COI;28S;16S	JQ611298,JQ611299,JQ611391,JQ611392,JQ611430,JQ611431
		Caryophyllia	*diomedeae*	CAR	caryop		COI;28S	HM018614,HQ439613
		Caryophyllia	*grandis*	CAR	caryop		28S	HQ439614
		Caryophyllia	*inornata*	CAR	caryop		12S;16S;28S	AF265599,EF597042,EU262777
		Caryophyllia	*lamellifera*	CAR	caryop		COI;28S	HM018616,HQ439616
		Caryophyllia	*planilamellata*	CAR	caryop		28S	HQ439617
		Caryophyllia	*quadragenaria*	CAR	caryop		28S	HQ439618
		Caryophyllia	*ralphae*	CAR	caryop		COI;28S	HM018617,HQ439619
		Caryophyllia	*rugosa*	CAR	caryop		COI;28S	HM018618,HQ439620
		Caryophyllia	*scobinosa*	CAR	caryop		28S	HQ439621
		Caryophyllia	*smithii*	CAR	caryop		COI;ITS;28S;16S	AF549216,JQ611300,JQ611301,JQ611393,JQ611394,JQ611432,JQ611433
		Caryophyllia	*transversalis*	CAR	caryop		28S	HQ439622
		Caryophyllia	*unicristata*	CAR	caryop		28S	HQ439623
		Caryophyllia	*versicolorata*	CAR	caryop		28S	HQ439624
	Ceratotrochus	*Ceratotrochus*	*magnaghii*	CAR	cerato		16S;28S	AF265597,EU262879
	Conotrochus	*Conotrochus*	*funicolumna*	CAR	conotr		COI;28S	HM018621,HQ439629
	Crispatotrochus	*Crispatotrochus*	*rugosus*	CAR	crispa		16S;28S	AF265600,EU262860
	Dasmosmilia	*Dasmosmilia*	*cf*	CAR	dasmos		COI	HM018625
		Dasmosmilia	*lymani*	CAR	dasmos		28S	HQ439631,HQ439632,HQ439633
	Desmophyllum	*Desmophyllum*	*dianthus*	CAR	desmop		COI;ITS;12S;16S;28S	AF549217,GQ868666,GQ868667,GQ868676,HM015305,HM015307,HM015308,HM015309,HM015310,JF827640,JF827641,JF827642,JQ611269,JQ611270,JQ611271,JQ611272,JQ611273,JQ611274,JQ611275,JQ611276,JQ611277,JQ611278,JQ611279,JQ611280,JQ611281,JQ611282,JQ611283,JQ611284,JQ611285,JQ611287,JQ611288,JQ611289,JQ611290,JQ611291,JQ611292,JQ611293,JQ611294,JQ611295,JQ611307,JQ611308,JQ611309,JQ611310,JQ611311,JQ611312,JQ611313,JQ611314,JQ611315,JQ611316,JQ611362,JQ611363,JQ611364,JQ611369,JQ611374,JQ611377,JQ611378,JQ611379,JQ611380,JQ611381,JQ611382,JQ611383,JQ611384,JQ611385,JQ611386,JQ611387,JQ611388,JQ611401,JQ611402,JQ611403,JQ611405,JQ611409,JQ611411,JQ611414,JO611415.JO611416.JO611417.JO611419.JO611421.JO611422
	Hoplangia	*Hoplangia*	*durotrix*	CAR	hoplan		12S;28S	EF597063,EF597064,EU262817,EU262824

Family	Genus	*Genus*	*Species*	Fam. code	Gen. code *	Genes	No. GenBank Access
	Lophelia	*Lophelia*	*pertusa*	CAR	lophel	CYTB;COI;ITS;12S;16S;18S;28S	AY257253,AY257254,AY257256,AY257257,AY257258,AY257260,AY257261,AY257262,AY257264,AY257265,AY257266,AY257267,AY257268,AY257269,AY257270,AY257271,AY257272,AY257273,AY257274,AY257275,AY257276,AY257278,AY257279,AY257280,AY257281,AY257286,AY257287,AY257288,AY257289,AY257290,AY257293,AY257294,AY257296,AY257297,AY257299,AY257300,AY257301,AY257302,AY257303,AY257304,AY257306,AY257309,AY257310,AY257311,AY257312,AY257313,AY257314,AY257315,AY257316,AY257317,AY257318,AY257319,AY257320,AY257321,AY257322,AY257323,AY257324,AY257325,AY257326,AY257327,AY257328,AY257329,AY257330,AY257331,AY257332,AY257333,AY257336,AY354196,AY354197,FR821799,JQ611296,JQ611428,KC875348,KC875349
	Paracyathus	*Paracyathus*	*pulchellus*	CAR	paracy	16S;28S	AF265603,EU262820
	Phyllangia	*Phyllangia*	*mouchezii*	CAR	phylla	12S;16S;28S	AF265605,AF549245,EF597022,EU262798
		Phyllangia	*papuensis*	CAR	phylla	COI;28S	HM018660,HQ439669
	Polycyathus	*Polycyathus*	*muellerae*	CAR	polycy	12S;16S;28S	AF265606,EF597026,EU262790
		Polycyathus	*sp*	CAR	polycy	CYTB;12S;16S;COI	JF825140
	Pourtalosmilia	*Pourtalosmilia*	*anthophyllites*	CAR	pourta	COI;28S	JQ611390,JQ611429
	Rhizosmilia	*Rhizosmilia*	*maculata*	CAR	rhizos	12S;16S;28S	AF265602,EF597023,EU262796
		Rhizosmilia	*robusta*	CAR	rhizos	COI;28S	HM018664,HQ439635
		Rhizosmilia	*sagamiensis*	CAR	rhizos	28S	HQ439636
	Stephanocyathus (Acinocyathus)	*Stephanocyathus*	*spiniger*	CAR	steaci	COI;28S	HM018665,HQ439638
	Stephanocyathus (Odontocyathus)	*Odontocyathus*	*weberianus*	CAR	steodo	16S	AF265594
		Stephanocyathus	*coronatus*	CAR	steodo	28S	HQ439637
		Stephanocyathus	*weberianus*	CAR	steodo	28S	EU262795
	Tethocyathus	*Tethocyathus*	*virgatus*	CAR	tethoc	28S	HQ439639
	Thalamophyllia	*Thalamophyllia*	*gasti*	CAR	thalam	12S;16S;28S	AF265590,EF597086,EU262788
	Trochocyathus	*Trochocyathus*	*efateensis*	CAR	trocho	COI;28S	HM018667,HQ439640
		Trochocyathus	*rhombocolumna*	CAR	trocho	COI;16S;28S	HM018668,HQ439641,HQ439642,HQ439703,HQ439704
	Vaughanella	*Vaughanella*	*concinna*	CAR	vaugha	12S;28S	EF596989,HQ439643,HQ439644
		Vaughanella	*sp*	CAR	vaugha	16S;28S	AF265595,HQ439645
Coscinaraeidae	*Anomastrea*	*Anomastrea*	*irregularis*	COS	anomas	COI	AM494869,AM494870
	Coscinaraea	*Coscinaraea*	*columna*	COS	coscin	CYTB;COI;ITS;18S	AB441210,AB441295,AB441406,AM494858,AM494859,HE978508
		Coscinaraea	*monile*	COS	coscin	CYTB	KJ573657,KJ573658
		Coscinaraea	*sp*	COS	coscin	28S	EU262826
		Craterastrea	*levis*	COS	coscin	COI;ITS;18S	HE978504,HE978505,HE978509,HE978510
	Horastrea	*Horastrea*	*indica*	COS	horast	COI	AM494864,AM494865
Deltocyathidae	*Deltocyathus*	*Deltocyathus*	*corrugatus*	DEL	deltoc	28S	JX486105
		Deltocyathus	*inusitatus*	DEL	deltoc	COI;28S	HM018626,JX486108
		Deltocyathus	*magnificus*	DEL	deltoc	COI;16S;28S	HM018627,HQ439634,HQ439769,HQ439770,HQ439771
		Deltocyathus	*ornatus*	DEL	deltoc	COI	HM018628
		Deltocyathus	*rotulus*	DEL	deltoc	COI	HM018629
		Deltocyathus	*sarsi*	DEL	deltoc	COI;28S	HM018630,JX486106
		Deltocyathus	*suluensis*	DEL	deltoc	COI;28S	HM018631,JX486109
		Deltocyathus	*vaughani*	DEL	deltoc	28S	JX486107

Family	Genus	*Genus*	*Species*	Fam. code	Gen. code	*	Genes	No. GenBank Access
Dendrophyliide	*Astroides*	*Astroides*	*calycularis*	DEN	astroi		28S;ITS;COI	AF549248,FM164499,JQ343061,JQ343062,JQ343063,JQ343064,JQ343065,JQ343066,JQ343067,JQ343068,JQ343069,JQ343070,JQ343071,JQ343072,JQ343073,JQ343074,JQ343075,JQ343076,JQ343077,JQ343078,JQ343079,JQ343080,JQ343081,JQ343082,JQ343083,JQ343084,JQ343085,JQ343086,JQ343087,JQ343088,JQ343089,JQ343090,JQ343091,JQ343092,JQ343093,JQ343094,JQ343095,JQ343096,JQ343097,JQ343098,JQ343099,JQ343100,JQ343101,JQ343102,JQ343103,JQ343104,JQ343105,JQ343106,JQ343107,JQ343108,JQ343109,JQ343110,JQ343111,JQ343112,JQ343113,JQ343114,JQ343115,JQ343116,JQ343117,JQ343118,JQ343119,JQ343120,JQ343121,JQ343122,JQ343123,JQ343124,JQ343125,JQ343126,JQ343127,JQ343128,JQ343129,JQ343130,JQ343131,JQ343132,JQ343133,JQ343134,JQ343135,JQ343136,JQ343137,JQ343138,JQ343139,JQ343140,JQ343141,JQ343142,JQ343143,JQ343144,JQ343145,JQ343146,JQ343147,JQ343148,JQ343149,JQ343150,JQ343151,JQ343152,JQ343153,JQ343154,JQ343155,JQ343156,JQ343157,JQ343158,JQ343159,JQ343160,JQ343161,JQ343162,JQ343163,JQ343164,JQ343165,JQ343166,JQ343167,JQ343168,JQ343169,JQ343170,JQ343171,JQ343172,JQ343173,JQ343174,JQ343175,JQ343176,JQ343177,JQ343178,JQ343179,JQ343180,JQ343181,JQ343182,JQ343183,JQ343184,JQ343185,JQ343186,JQ343187,JQ343188,JQ343189,JQ343190,JQ343191,JQ343192
	Balanophyllia	*Balanophyllia*	*cf*	DEN	balano		16S;28S	HQ439646,HQ439709
		Balanophyllia	*cornu*	DEN	balano		COI;28S;16S	HM018605,HQ439647,HQ439710
		Balanophyllia	*desmophyllioides*	DEN	balano		COI;28S;16S	HM018607,HQ439648,HQ439711
		Balanophyllia	*elegans*	DEN	balano		ITS;COI	AF180111,DQ445805
		Balanophyllia	*europaea*	DEN	balano		ITS;28S	AF549220
		Balanophyllia	*gigas*	DEN	balano		28S;16S	HQ439649,HQ439712
		Balanophyllia	*regia*	DEN	balano		28S;16S;12S	AF265587,AF549221,EF597047,EU262813
		Balanophyllia	*sp*	DEN	balano		COI;12S;16S;28S	EF597048,EU262855,HM018606,HM018608,HM018609,HM018610,HM018611,HM018612,HQ439650,HQ439651,HQ439652,HQ439653,HQ439713,HQ439714,HQ439715,HQ439716
	Cladopsammia	*Cladopsammia*	*gracilis*	DEN	cladops		ITS;28S	DQ533612,DQ533613,DQ533614,DQ533615,DQ533616,DQ533617,DQ533618,DQ533619,DQ533620,EU262774
		Dendrophyllia	*gracilis*	DEN	cladops		16S	AF265588
	Dendrophyllia	*Dendrophyllia*	*alternata*	DEN	dendro		16S	AF550366
		Dendrophyllia	*sp*	DEN	dendro		CYTB;COI;16S	AB441239,AB441324,JX629248
	Eguchipsammia	*Eguchipsammia*	*fistula*	DEN	eguchi		16S	JX629250
	Enallopsammia	*Enallopsammia*	*rostrata*	DEN	enallo		COI;ITS;16S;28S	HM015295,HM015296,HM015297,HM015298,HM015299,HM015300,HM018632,HQ439654,HQ439717,HQ439718,U40294
	Endopachys	*Endopachys*	*grayi*	DEN	endopa		16S;28S	HQ439655,HQ439719
	Heteropsammia	*Heteropsammia*	*sp*	DEN	hetero		12S;28S	EF597050,EU262866
	Leptopsammia	*Leptopsammia*	*pruvoti*	DEN	leptop		12S;16S;18S	AF265579,EF597068,EU262851
	Tubastraea	*Tubastraea*	*aurea*	DEN	tubast	*	CYTB;COI;ITS;12S	AB441235,AB441236,AB441237,AB441320,AB441321,AB441322,AF333064,AY722796,JQ920444
		Tubastraea	*coccinea*	DEN	tubast		COI;ITS;12S;16S;28S	AF180110,DQ445806,DQ445807,EF597045,EF597046,EU262854,EU262864,L76022
		Tubastraea	*micranthus*	DEN	tubast		28S	AF549219
		Tubastraea	*sp*	DEN	tubast	*	CYTB;COI;ITS	AB441238,AB441323,DQ533621,HQ420828
		Turbinaria	*irregularis*	DEN	turbin		28S	JQ966136
		Turbinaria	*mesenterina*	DEN	turbin		12S	AF333063
		Turbinaria	*peltata*	DEN	turbin		CYTB;COI;12S;16S;28S	AB441240,AB441325,AB907099,AB907100,AB907101,AF265609,EF597043,EF597044,EU262799,JQ966131,L76023
Diploastreidae	*Diploastrea*	*Diploastrea*	*heliopora*	DIP	diploa		CYTB;COI;ITS;18S;28S	AB117290,AB117375,AB441396,AB441397,AF549227,AY509701,AY509702,AY509703,AY509704,AY509707,AY509708,AY509709,AY509710,AY509711,AY509717,AY509719,AY509720,AY509721,AY509722,AY509723,AY509724,AY509725,AY509726,AY509727,AY509728,AY509729,AY509730,AY509731,AY509732,AY509733,AY509734,AY509735,AY509736,AY509737,AY509738,AY509739,EU371660,EU371661,HQ203315,HQ203410,KJ573663,KJ573664
Euphylliidae	*Catalaphyllia*	*Catalaphyllia*	*jardinei*	EUP	catala		12S;28S	EF596997,EU262874
	Ctenella	*Ctenella*	*chagius*	EUP	ctenel		CYTB;COI	AB441208,AB441293
	Euphyllia	*Euphyllia*	*ancora*	EUP	euphyl		CYTB;COI;12S;16S	AB441204,AB441205,AB441289,AB441290,AF265598,EF597097,EF597098,JF825139,L76002
		Euphyllia	*divisa*	EUP	euphyl		CYTB	AB441288
		Euphyllia	*glabrescens*	EUP	euphyl		CYTB;COI	AB441206,AB441207,AB441291,AB441292,HQ420830
	Galaxea	*Achrelia*	*horrescens*	EUP	galaxe	*	16S	L75994

Family	Genus	*Genus*	*Species*	Fam. code	Gen. code	*	Genes	No. GenBank Access
		Achrelia	*sp*	EUP	galaxe	*	28S	JQ966156
		Galaxea	*astreata*	EUP	galaxe	*	COI;12S;16S:28S	AF333056,JQ347819,JQ347842,JQ920457,JQ966143
		Galaxea	*fascicularis*	EUP	galaxe		CYTB;COI;ITS;12S;16S;18S;28S	AB441201,AB441202,AB441286,AB441287,AF263360,HQ420829,JQ347818,JQ347841,JQ920441,JQ966129, L76006.AY722764.AY722765
		Galaxea	*horrescens*	EUP	galaxe		12S	EF597096
	undefined genus	*Galaxea*	*sp*	EUP	galxxx		16S	JQ347820
Fungiacyathidae	*Fungiacyathus (Bathvactis)*	*Fungiacyathus*	*marenzelleri*	FGY	bathya	*	12S;16S;28S	AF550364,EF589061,EF597074,EU262862,L76004
		Fungiacyathus	*turbinolioides*	FGY	bathya	*	COI;16S;28S	HM018648,HQ439679,HQ439759
	undefined genus	*Fungiacyathus*	*sp*	FGY	fgyxxx		CYTB;COI;ITS;18S	AB441255,AB441340,AY722757,AY722758
	Fungiacyathus (Fungiacyathus)	*Fungiacyathus*	*fragilis*	FGY	fungcy		COI;16S;28S	HM018645,HQ439675,HQ439753
		Fungiacyathus	*pusillus*	FGY	fungcy		COI;16S;28S	HM018646,HQ439677,HQ439754,HQ439755
		Fungiacyathus	*pusilluspacificus*	FGY	fungcy		28S	HQ439676
		Fungiacyathus	*stephanus*	FGY	fungcy		CYTB;COI;12S;16S;28S	HM018647,HQ439678,HQ439758,JF825138
Flabellidae	*Flabellum (Flabellum)*	*Flabellum*	*arcuatile*	FLA	flabel		COI;16S;28S	HM018636,HQ439657,HQ439721,HQ439722
		Flabellum	*curvatum*	FLA	flabel		28S	EU262783
		Flabellum	*folkesoni*	FLA	flabel		COI;16S;28S	HM018639,HQ439660,HQ439725
		Flabellum	*impensum*	FLA	flabel		16S;28S	AF265582,EU262846
		Flabellum	*lamellulosum*	FLA	flabel		COI;16S;28S	HM018640,HQ439661,HQ439726
		Flabellum	*magnificum*	FLA	flabel		16S;28S	AB510167,AB510179,HQ439663
		Flabellum	*pavoninum*	FLA	flabel		16S;28S	AB510168,AB510180
		Flabellum	*vaughani*	FLA	flabel		COI;28S	HM018644,HQ439665
	Flabellum (Ulocyathus)	*Flabellum*	*angulare*	FLA	flaulo		16S	AF550363
		Flabellum	*apertum*	FLA	flaulo		COI;16S;28S	HM018635,HQ439656,HQ439720
		Flabellum	*deludens*	FLA	flaulo		COI;16S;28S	AB510170,AB510171,AB510183,AB510184,HM018638,HQ439659,HQ439724
		Flabellum	*japonicum*	FLA	flaulo		16S;28S	AB510169,AB510178,AB510181,AB510182
		Flabellum	*lowekeyesi*	FLA	flaulo		COI;16S;28S	HM018641,HQ439662,HQ439728,HQ439729,HQ439731
		Flabellum	*tuthilli*	FLA	flaulo		COI;16S;28S	HM018643,HQ439664,HQ439733,HQ439734
	undefined genus	*Flabellum*	*cf*	FLA	flaxxx		COI;16S;28S	HM018637,HQ439658,HQ439723
		Flabellum	*sp*	FLA	flaxxx		COI	HM018642
	Javania	*Javania*	*cailleti*	FLA	javani		COI;ITS;16S;28S	JQ611306,JQ611355,JQ611399,JQ611438
		Javania	*fusca*	FLA	javani		COI;16S;28S	HM018652,HQ439666,HQ439737,HQ439738
		Javania	*insignis*	FLA	javani		16S;28S	AB510174,AB510187
		Javania	*lamprotichum*	FLA	javani		COI;16S;28S	HM018653,HQ439667,HQ439740,HQ439741
		Javania	*sp*	FLA	javani		COI;16S;28S	HM018654,HQ439668,HQ439743
	Monomyces	*Monomyces*	*pygmaea*	FLA	monomy		16S	AF265583
	Placotrochus	*Placotrochus*	*laevis*	FLA	placot		16S	AF265589,AF265604
		Placotrochus	*sp*	FLA	placot		12S;28S	EF597028,EF597071,EU262841
	Placotrochides	*Placotrochides*	*scaphula*	FLA	plactrh		COI;16S;28S	HM018661,HQ439670,HQ439744,HQ439745
	Rhizotrochus	*Rhizotrochus*	*flabelliformis*	FLA	rhizot		12S;28S	EF597070,EU262812
		Rhizotrochus	*typus*	FLA	rhizot		16S;28S	AB510175,AB510176,AB510177,AB510188
	Truncatoflabellum	*Truncatoflabellum*	*australiensis*	FLA	trunca		COI;16S;28S	HM018670,HQ439671,HQ439746,HQ439747,HQ439748
		Truncatoflabellum	*candeanum*	FLA	trunca		COI;28S	HM018671,HQ439672
		Truncatoflabellum	*formosum*	FLA	trunca		16S;28S	HQ439673,HQ439674,HQ439752
		Truncatoflabellum	*macroeschara*	FLA	trunca		COI	HM018672
		Truncatoflabellum	*sp*	FLA	trunca		COI;12S	EF596985,HM018673,HM018674
		Truncatoflabellum	*spheniscus*	FLA	trunca		16S;28S	AB510172,AB510173,AB510185,AB510186
Fungiidae	*Ctenactis*	*Ctenactis*	*albitentaculata*	FUN	ctenac		COI;ITS	EU149813,EU149869
		Ctenactis	*crassa*	FUN	ctenac		COI;ITS	EU149814,EU149815,EU149859,EU149889
		Ctenactis	*echinata*	FUN	ctenac		COI;ITS	EU149816,EU149817,EU149879,EU149899

Family	Genus	*Genus*	*Species*	Fam. code	Gen. code	*	Genes	No. GenBank Access
	Cycloseris	*Cycloseris*	*costulata*	FUN	cyclos		COI;ITS	EU149818,EU149819,EU149870,EU149890,EU202718
		Cycloseris	*cyclolites*	FUN	cyclos		COI;ITS	EU149821,EU202719
		Cycloseris	*fragilis*	FUN	cyclos		COI	EU149860,EU149880
		Cycloseris	*sinensis*	FUN	cyclos		COI;ITS	EU149822,EU149900
		Cycloseris	*sp*	FUN	cyclos		COI;ITS;18S	HE599790,HE599791,HE599792,HE599793,HE599794,HE599795,HE599796,HE599797,HE599798,HE599799,HE599800,HE599801,HE599802,HE599803,HE600143,HE600154
		Cycloseris	*tenuis*	FUN	cyclos		COI;ITS	EU149823,EU149871,EU149891
		Cycloseris	*vaughani*	FUN	cyclos		COI;ITS	EU149824,EU149861,EU149881
		Coscinaraea	*wellsi*	FUN	cyclos	*	COI	AM494861,AM494862
		Fungia	*fragilis*	FUN	cyclos	*	16S	L75998
		Fungia	*vaughani*	FUN	cyclos	*	16S	L75999
		Lithophyllon	*mokai*	FUN	cyclos	*	COI;ITS	EU149842,EU149877,EU149897,EU149906
		Psammocora	*explanulata*	FUN	cyclos	*	COI	AM494845,AM494846,AM494879
	Danafungia	*Danafungia*	*scruposa*	FUN	danafu		COI	EU149872
		Fungia	*horrida*	FUN	danafu	*	ITS	EU149826
		Fungia	*scruposa*	FUN	danafu	*	ITS	EU149827,EU149828
	Fungia	*Fungia*	*fungites*	FUN	fungia		CYTB;COI;ITS	EU149829,EU149892,KJ573672,KJ573673
	undefined genus	*Fungia*	*sp*	FUN	funxxx	*	COI;ITS;12S	EF596986,EF596987,EU149851,EU149913
	Halomitra	*Halomitra*	*clavator*	FUN	halomi		COI;ITS;18S	EU149837,EU149904
		Halomitra	*pileus*	FUN	halomi		COI;ITS;18S	AM494873,AM494874,EU149838,EU149865,EU149875,EU149895
	undefined genus	*Halomitra*	*sp*	FUN	halxxx	*	12S;28S	EF596988,EU262797
	Heliofungia	*Fungia*	*fralinae*	FUN	heliof	*	ITS	EU149825
		Heliofungia	*actiniformis*	FUN	heliof		COI;ITS;12S;28S	AM849553,AM849555,AM849562,AM849563,AM849564,AM849565,AM849567,AM849568,AM849569,EF596995,EU149839,EU149876,EU149885,EU149905,EU202720,EU262775
		Heliofungia	*fralinae*	FUN	heliof		COI	EU149901
		Heliofungia	*sp*	FUN	heliof		COI	HM018650
	Herpolitha	*Herpolitha*	*limax*	FUN	herpol		CYTB;COI;ITS	AB441223,AB441308,AM494877,AM494878,EU149840,EU149841,EU149866
	undefined genus	*Herpolitha*	*sp*	FUN	herxxx	*	12S;28S	EF596994,EU262811
	Lithophyllon	*Fungia*	*concinna*	FUN	lithop	*	ITS	EU149832
		Fungia	*scabra*	FUN	lithop	*	ITS	EU149833
		Fungia	*spinifer*	FUN	lithop	*	ITS	EU149834
		Lithophyllon	*undulatum*	FUN	lithop		COI;ITS	EU149843,EU149844,EU149867,EU149887
		Verrillofungia	*concinna*	FUN	lithop	*	COI	EU149893,EU202721
		Verrillofungia	*repanda*	FUN	lithop	*	COI	EU149863,EU149883
		Verrillofungia	*scabra*	FUN	lithop	*	COI	EU149874,EU149903
		Verrillofungia	*spinifer*	FUN	lithop	*	COI	EU149864
	Lobactis	*Lobactis*	*scutaria*	FUN	lobact		CYTB;COI;28S	AB441224,AB441309,EU149862,EU149873,EU149882,EU149902,EU262850,HM048841
		Fungia	*scutaria*	FUN	lobact	*	ITS;12S;16S	DQ320497,EU149830,EU149831,L76005
	Pleuractis	*Fungia*	*granulosa*	FUN	pleura	*	ITS	EU149835,EU149836
		Fungia	*gravis*	FUN	pleura	*	ITS	EU149848
		Fungia	*moluccensis*	FUN	pleura	*	18S; ITS	EU149849
		Fungia	*paumotensis*	FUN	pleura	*	COI;ITS	AM494875,AM494876,EU149850
		Fungia	*taiwanensis*	FUN	pleura	*	ITS	EU149852
		Pleuractis	*granulosa*	FUN	pleura		COI	EU149884
		Pleuractis	*gravis*	FUN	pleura		COI	EU149910
		Pleuractis	*moluccensis*	FUN	pleura		COI	EU149909
		Pleuractis	*paumotensis*	FUN	pleura		COI	EU149911,EU149912
	Podabacia	*Podabacia*	*crustacea*	FUN	podaba		COI;ITS;18S	AM494871,AM494872,EU149845,EU149878,EU149907
		Podabacia	*motuporensis*	FUN	podaba		COI;ITS	EU149846,EU149868
	Polyphyllia	*Polyphyllia*	*sp*	FUN	polyph		12S;28S	EF596993,EU262880
		Polyphyllia	*talpina*	FUN	polyph		COI;ITS;18S	EU149853,EU149915
	Polyphyllia	*Polyphyllia*	*dentata*	FUN	sandol		COI;ITS	EU149854,EU149855,EU149856,EU149914,EU149918
		Sandalolitha	*robusta*	FUN	sandol		CYTB;COI;ITS;18S	AB441411,EU149857,EU149917,KJ573692,KJ573693

Family	Genus	*Genus*	*Species*	Fam. code	Gen. code	*	Genes	No. GenBank Access
		Sandalolitha	*sp*	FUN	sandol		12S;28S	EF596991,EU262857
	undefined genus	*Podabacia*	*sp*	FUN	xxxxxx	*	COI;ITS	EU149847,EU149888
	Zoopilus	*Zoopilus*	*echinatus*	FUN	zoopil		COI;ITS:12S;16S;28S	EF596990,EU149858,EU149916,EU262870,L76024
Gardineridae	*Gardineria*	*Gardineria*	*hawaiiensis*	GAR	gardin		COI;12S;16S:28S	GQ868657,GQ868658,GQ868659,GQ868660,GQ868673,GQ868677,GQ868678,GQ868679,GQ868680,GQ868687.GO868699.GO868701.GO868702
		Gardineria	*paradoxa*	GAR	gardin		COI;12S;16S:28S	GQ868656,GQ868671,GQ868681,GQ868682,GQ868698,GQ868700
		Gardineria	*sp*	GAR	gardin		16S;18S	GQ868675,GQ868686
Gyuniidae		*Guynia*	*annulata*	GUY	guynia		16S;28S	AF265580,AF549233
Lobophyllidae	*Acanthastrea*	*Acanthastrea*	*bowerbanki*	LOB	acanth		COI	HF954208,HF954209
		Acanthastrea	*echinata*	LOB	acanth		COI;CYTB;18S;ITS;28S	AB117249,AB117250,AB117326,AB117327,AB441401,AY722739,AY722740,EU371658,HE648539,HE648540,HE648541,HE654623,HE654624,HE654625,HF954214,HF954215,HF954300,HF954301,HF954302,HQ203308.HO203399.KJ573645.KJ573646
		Acanthastrea	*faviaformis*	LOB	acanth		COI;18S	HF954212,HF954299
		Acanthastrea	*hemprichii*	LOB	acanth		COI;18S	HF954220,HF954221,HF954308,HF954309,HF954310
		Acanthastrea	*hillae*	LOB	acanth		COI;CYTB	AB441199,AB441284,HF954206
		Acanthastrea	*ishigakiensis*	LOB	acanth		COI;18S	HF954205,HF954292
		Acanthastrea	*maxima*	LOB	acanth		18S;ITS;COI	HE648542,HE648543,HE648544,HE654626,HE654627,HE654628,HF954210,HF954211
		Acanthastrea	*rotundaflora*	LOB	acanth		COI;18S	HF954216,HF954217,HF954238,HF954303,HF954304,HF954306
		Acanthastrea	*rotundata*	LOB	acanth		COI;CYTB	AB117251,AB117328
		Acanthastrea	*subechinata*	LOB	acanth		18S	HF954307
	Cynarina	*Cynarina*	*lacrimalis*	LOB	cynari		CYTB;COI;12S;18S;28S	EF597034,EU262825,AB117246,AB117323,HE648552,HE654636,HF954201,HF954288
		Cynarina	*sp*	LOB	cynari		16S	AF265613
	Echinophyllia	*Echinophyllia*	*aspera*	LOB	echino		CYTB;COI;ITS;18S;28S	AB117252,AB117329,AB441400,AF549241,HE648564,HE654648,HF954242,HF954244,HF954245,HF954329,HF954330.HF954331,HF954332
		Echinophyllia	*echinata*	LOB	echino		18S; ITS	HF954232,HF954321,HF954322
		Echinophyllia	*echinoporoides*	LOB	echino		CYTB;COI;ITS;18S;	AB117331,HF954235,HF954236,HF954323,HF954324,HF954326
		Echinophyllia	*orpheensis*	LOB	echino		CYTB;COI;ITS;12S;18S	AB117253,AB117330,AF333065,HF954327,HF954328
		Echinophyllia	*sp*	LOB	echino		COI;ITS;12S;18S	EF596999,HF954229,HF954230,HF954231,HF954317,HF954318,HF954319
	Lobophyllia	*Lobophyllia*	*cf*	LOB	lobop	*	COI;ITS;18S	HE648553,HE648553,HE654637
		Lobophyllia	*corymbosa*	LOB	lobop		CYTB;COI;18S;28S	AB117241,AB117318,AF549237,HE648554,HE654638,HF954254,HF954255,HF954341,HF954342
		Lobophyllia	*costata*	LOB	lobop	*	COI;18S	HF954246,HF954247,HF954333,HF954334
		Lobophyllia	*diminuta*	LOB	lobop		COI;18S	HF954252,HF954253,HF954339,HF954340
		Lobophyllia	*flabelliformis*	LOB	lobop		COI;ITS;18S	HF954248,HF954249,HF954335,HF954336
		Lobophyllia	*hemprichii*	LOB	lobop		CYTB;COI;ITS;12S;16S;18S;28S	AB117240,AB117317,EF597013,EU262833,HE648555,HE648556,HE654639,HE654640,HF954256,HF954343,KJ573676.KJ573677.L76013
		Lobophyllia	*pachysepta*	LOB	lobop		CYTB;COI	AB117242,AB117319
		Lobophyllia	*robusta*	LOB	lobop		COI;18S	HF954250,HF954251,HF954337,HF954338
	Micromussa	*Micromussa*	*amakusensis*	LOB	microm		CYTB;COI;ITS;18S	AB441200,AB441285,AB441403,HE654641,HE654642,HE654643,HF954198
	Moseleya	*Moseleya*	*latistellata*	LOB	mosele		COI;ITS;18S;28S	HQ203293,HQ203376,HQ203376,HQ203493
	Oxypora	*Oxypora*	*glabra*	LOB	oxypor		COI;ITS	HF954223,HF954224,HF954225,HF954311,HF954312,HF954313
		Oxypora	*lacera*	LOB	oxypor		CYTB:COI;ITS;18S	AB117255,AB117332,AB117333,HF954226,HF954227,HF954314,HF954315,HF954316
		Oxypora	*sp*	LOB	oxypor		12S;28S	EF597015,EU262876
	Parascolymia	*Parascolymia*	*vitiensis*	LOB	parasc		CYTB;COI;18S	AB117247,AB117324,HF954202,HF954203,HF954204,HF954289,HF954290,HF954291
	Symphyllia	*Symphyllia*	*agaricia*	LOB	symphy		COI;18S	HF954263,HF954350,HF954351
		Symphyllia	*erythraea*	LOB	symphy		COI;18S	HF954257,HF954258,HF954259,HF954344,HF954345,HF954346
		Symphyllia	*radians*	LOB	symphy		COI;ITS;18S	HE648560,HE648561,HE648562,HE648563,HE654644,HE654645,HE654647,HF954265,HF954266,HF954352,HF954353
		Symphyllia	*recta*	LOB	symphy		COI;18S	HF954267,HF954268,HF954354,HF954355
		Symphyllia	*sp*	LOB	symphy		COI	HQ420831
		Symphyllia	*valenciennesii*	LOB	symphy		COI;18S	HF954260,HF954262,HF954347,HF954348,HF954349,HM018666
Meandrinidae	*Dendrogyra*	*Dendrogyra*	*cylindrus*	MEA	dengyr		CYTB;COI;28S	AB117299,AB117384,AF549249,EU262819,KJ573659,KJ573660
	Dichocoenia	*Dichocoenia*	*stokesi*	MEA	dichoc		CYTB;COI;16S;28S	AB117383,AF265607,AF549235,AY451360,EU262778,EU262875,KJ573661,KJ573662
	Eusmilia	*Eusmilia*	*fastigiata*	MEA	eusmil		CYTB;COI;12S;28S	AB117294,AB117380,AF549218,EF597018,EF597019,EU262771,EU262872,KJ573667

Family	Genus	*Genus*	*Species*	Fam. code	Gen. code	*	Genes	No. GenBank Access
		Eusmilia	*sp*	MEA	eusmil		COI	AY451345
	Meandrina	*Meandrina*	*braziliensis*	MEA	meandr	*	CYTB;COI	AB117297,AB117382
		Meandrina	*meandrites*	MEA	meandr		CYTB;COI;12S;28S	AB117295,AB117296,AB117381,AY451361,AY451362,EF597032,EU262815
	undefined genus	*Meandrina*	*pectinata*	MEA	xxxxme	*	28S	AF549234
Merulinidae	*Astrea*	*Montastraea*	*cfannuligera*	MER	astrea	*	COI	JN248781
		Montastraea	*curta*	MER	astrea	*	CYTB;ITS;28S	AB117359,FR838000,HQ203483,HQ203484
		Montastrea	*curta*	MER	astrea	*	COI;ITS;18S;28S	AF549230,AY722774,AY722775,EU371706
		Phymastrea	*curta*	MER	astrea	*	CYTB	KJ573682,KJ573683
	Caulastraea	*Caulastraea*	*echinulata*	MER	caulas		28S	HQ203401
		Caulastraea	*furcata*	MER	caulas		CYTB;12S;16S;28S	AB117355,AF549224,EF597035,HQ203402,L75997
	Coelastrea	*Goniastrea*	*aspera*	MER	coelas	*	CYTB;COI;18S;28S	AB117271,AB117351,AY722759,AY722760,AY722761,FJ345430,HQ203354,HQ203458
		Goniastrea	*palauensis*	MER	coelas	*	COI;ITS;18S;28S	AY722766,AY722767,AY722768,EU371699,HQ203466
	Cyphastrea	*Cyphastrea*	*chalcidicum*	MER	cyphas		CYTB;ITS;18S;28S	AB117336,HQ203311,HQ203312,HQ203404,HQ203405
		Cyphastrea	*japonica*	MER	cyphas		18S; ITS	AY722749,AY722750,AY722751
		Cyphastrea	*microphthalma*	MER	cyphas		ITS;18S;28S	HE648471,HE648472,HE648473,HE648474,HQ203406
		Cyphastrea	*ocellina*	MER	cyphas		12S;16S	EF596996,L76132
		Cyphastrea	*serailia*	MER	cyphas		CYTB;ITS;18S;28S	AB117334,AB117335,HQ203313,HQ203407,HQ203408,HQ203409,JQ966140
	Dipsastraea	*Barabattoia*	*amicorum*	MER	dipsas	*	COI;28S;18S;ITS;CYTB	AB441193,AB441278,FJ345412,FJ345413,HQ203309,HQ203400
		Favia	*danae*	MER	dipsas	*	COI;28S	EU371662,EU371663,FJ345423,HQ203416,HQ203417
		Favia	*favus*	MER	dipsas	*	CYTB;COI;12S;18S;28S	AB117267,AB117346,AF177048,EU371664,EU371665,EU371710,FJ345424,HE654563,HQ203255,HQ203256,HQ203257,HQ203322,HQ203323,HQ203324,HQ203325,HQ203418,HQ203419,HQ203420,HQ203421,HQ203422.HO203423
		Favia	*lizardensis*	MER	dipsas	*	COI;ITS;28S	EU371668,HM018633,HQ203328,HQ203426,HQ203427,HQ203428
		Favia	*matthai*	MER	dipsas	*	COI;ITS;18S;28S	HE648480,HE648481,HE648482,EU371669,EU371670,EU371671,EU371672,EU371673,HE654564,HE654565,HE654566,HQ203259,HQ203330,HQ203331,HQ203332,HQ203333,HQ203430,HQ203431,HQ203432,HQ203433
		Favia	*maxima*	MER	dipsas	*	COI;ITS;28S	EU371674,FJ345426,HQ203260,HQ203334,HQ203435
		Favia	*pallida*	MER	dipsas	*	CYTB;COI;ITS;18S;28S	AB117265,AB117266,AB117344,AB117345,EU371675,EU371676,HE648483,HE648484,HE648485,HE648486,HE648487,HE648488,HE648489,HE654567,HE654568,HQ203337,HQ203437,HQ203438
		Favia	*rosaria*	MER	dipsas	*	COI;ITS;28S	HQ203262,HQ203338,HQ203439
		Favia	*rotumana*	MER	dipsas	*	COI;ITS;18S;28S	FJ345427,FJ345428,HE648490,HE648491,HE648492,HE648493,HE648494,HE648496,HE654574,HQ203440,JO920464
		Favia	*speciosa*	MER	dipsas	*	CYTB;COI;ITS;18S;28S	AB441194,AB441279,EU371677,EU371678,EU371679,EU371680,EU371681,EU371682,EU371683,EU371684,EU371685,EU371686,HQ203264,HQ203341,HQ203342,HQ203443,HQ203444,HQ203445,JQ920465,JX120567
		Favia	*truncatus*	MER	dipsas	*	COI;ITS;28S	HM018634,HQ203266,HQ203344,HQ203447
	Echinopora	*Echinopora*	*gemmacea*	MER	echipo		CYTB;COI;ITS;18S;28S	AB117263,AB117342,HE648477,HE648478,HE654561,HE654562,HQ203316,HQ203411
		Echinopora	*horrida*	MER	echipo		COI;18S:28S	HQ203253,HQ203317,HQ203412
		Echinopora	*lamellosa*	MER	echipo		COI;ITS;12S;16S;28S	AF265586,EF597052,EU262773,FJ345419,HQ203318,HQ203413,L76003
		Echinopora	*mammiformis*	MER	echipo		ITS;18S;28S	HQ203319,HQ203414
		Echinopora	*pacificus*	MER	echipo		CYTB;COI;ITS;18S;28S	AB117261,AB117262,AB117340,AB117341,FJ345420,HQ203320,HQ203415
	Favites	*Favia*	*rotundata*	MER	favite	*	COI;28S	HQ203263,HQ203441,HQ203442
		Favites	*abdita*	MER	favite	*	COI;12S;18S;28S	AF263362,AF333060,AY722755,AY722756,EU371687,HE654582,HQ203448
		Favites	*chinensis*	MER	favite	*	CYTB;COI;28S	AB117269,AB117349,HQ203268,HQ203449
		Favites	*complanata*	MER	favite	*	COI;ITS;18S;28S	EU371689,EU371690,EU371691,EU371692,HE648499,HE648500,HE648501,HE648502,HE654583,HE654585,HE654586,HQ203450
		Favites	*flexuosa*	MER	favite	*	COI;28S	HQ203269,HQ203451
		Favites	*halicora*	MER	favite	*	CYTB;COI;28S	AB117268,AB117347,AB117348,HE654587,HE654588,HE654590,HQ203452
		Favites	*paraflexuosa*	MER	favite	*	COI;28S	EU371694,HQ203453
		Favites	*pentagona*	MER	favite	*	18S;28S	HE648511,HQ203351,HQ203454,HQ203455
		Favites	*russelli*	MER	favite	*	COI;28S	HQ203272,HQ203456
		Favites	*stylifera*	MER	favite	*	COI;ITS;28S	HQ203273,HQ203353,HQ203457
		Montastraea	*colemani*	MER	favite	*	COI;28S	HQ203284,HQ203482

Family	Genus	*Genus*	*Species*	Fam. code	Gen. code	*	Genes	No. GenBank Access
		Montastraea	*magnistellata*	MER	favite	*	CYTB;COI;28S	AB117279,AB117360,HQ203287,HQ203288,HQ203485,HQ203486
		Montastraea	*multipunctata*	MER	favite	*	28S	HQ203487
		Montastraea	*valenciennesi*	MER	favite	*	CYTB;COI;ITS;12S;18S;28S	AB117280,AB117361,AF333061,EU371713,EU371714,EU371715,EU371716,EU371717,EU371718,EU371719,EU371720,HQ203291,HQ203374,HQ203375,HQ203489,HQ203490,HQ203491,HQ203492
	undefined genus	*Favites*	*sp*	MER	fvtxxx	*	12S;16S	JQ347821,JQ347843
	Goniastrea	*Favia*	*stelligera*	MER	gonias	*	CYTB;COI;ITS;18S;28S	AB117264,AB117343,HQ203265,HQ203343,HQ203446,KJ573670,KJ573671
		Goniastrea	*australensis*	MER	gonias		COI;ITS;18S;28S	FJ345431,HQ203274,HQ203355,HQ203356,HQ203459,HQ203460,HQ203461
		Goniastrea	*deformis*	MER	gonias		CYTB;COI	AB441195,AB441280
		Goniastrea	*edwardsi*	MER	gonias		COI;ITS;28S	EU371697,HQ203357,HQ203462,HQ203463
		Goniastrea	*favulus*	MER	gonias		COI;ITS;28S	EU371698,FJ345433,HQ203358,HQ203464
		Goniastrea	*pectinata*	MER	gonias		CYTB;COI;ITS;28S	AB117270,AB117350,FJ345434,HQ203360,HQ203467,HQ203468,HQ203469
		Goniastrea	*retiformis*	MER	gonias		COI;ITS;12S;28S	EF597033,EU371700,EU371701,FR837981,FR837982,FR837991,FR837992,FR837993,HQ203275,HQ203361,HQ203470,HQ203471
	undefined genus	*Goniastrea*	*sp*	MER	gonxxx	*	ITS;18S	AY722762,AY722763
	Hydnophora	*Hydnophora*	*exesa*	MER	hydnop		CYTB;COI;ITS;12S;16S;18S;28S	AB117285,AB117370,AF333059,AY722769,AY722770,AY722771,HE648536,HE648537,HE648538,HE654621,HE654622.HO203276.HO203362.HO203472.JO347824.JO347846
		Hydnophora	*grandis*	MER	hydnop		CYTB;COI	AB117286,AB117371
		Hydnophora	*microconos*	MER	hydnop		COI;28S	HQ203277,HQ203473
		Hydnophora	*pilosa*	MER	hydnop		COI;28S	HQ203278,HQ203474
		Hydnophora	*rigida*	MER	hydnop		12S;16S;28S	EF597000,EU262858,L76009
	Leptoria	*Leptoria*	*irregularis*	MER	leptor		CYTB;COI;28S	AB117272,AB117352,AB117353,HQ203279,HQ203475
		Leptoria	*phrygia*	MER	leptor		CYTB;COI;12S;16S;18S;28S	AB117273,AB117354,AF549228,EF597051,EU371705,HE648527,HE654611,HQ203365,HQ203476,L76011
	Merulina	*Merulina*	*ampliata*	MER	meruli		COI;12S;28S	AF333058,HQ203280,HQ203477
		Merulina	*scabricula*	MER	meruli		CYTB;COI;16S;28S	AB117284,AB117369,HQ203281,HQ203478,L76014
	undefined genus	*Montastraea*	*cf*	MER	monxxx	*	28S	HQ203481
	Mycedium	*Mycedium*	*elephantotus*	MER	mycedi		CYTB;12S;18S;28S	AB117366,AB117367,AB126750,AF333057,HQ203377,HQ203494
		Mycedium	*robokaki*	MER	mycedi		ITS;18S;28S	HQ203378,HQ203495
		Mycedium	*sp*	MER	mycedi		12S;16S	AF265608,EF597036
	Orbicella	*Montastraea*	*annularis*	MER	orbice	*	CYTB;COI;ITS;12S;16S;18S;28S	AB065315,AB065317,AB065318,AB065319,AB065320,AB065321,AB065322,AB065323,AB065325,AB065326,AB065327,AB065329,AB065330,AB065331,AB065332,AB065333,AB117260,AB117337,AF549229,AP008973,AP008973,AP008973,AP008974,AP008974,AP008974,AY451352,EF597009,HQ203282,HQ203367,HQ203479
		Montastraea	*faveolata*	MER	orbice	*	CYTB;COI;ITS;12S;16S;28S	AB065312,AB065313,AB065314,AB065350,AB065353,AB065355,AB065360,AB065361,AB065362,AB117339,AP008977,AP008978,AY064532,AY451357,EF597008,EU262781,KF579904,KF579905,KF579906,KF579907.KF579908.KJ573684.KJ573685.U59906
		Montastraea	*franksi*	MER	orbice	*	CYTB;COI;ITS;12S;16S;28S	AB065301,AB065335,AB065339,AB065340,AB065343,AB065345,AB065346,AB065347,AB065348,AB065349,AP008975,AP008976,AY026375,EF597010,EU262849,KF579909,KJ482947
		Montastrea	*annularis*	MER	orbice	*	COI;ITS	AF013731,AF013737
		Montastrea	*faveolata*	MER	orbice	*	COI;ITS;18S	AF013733,AF013738
	Oulophyllia	*Oulophyllia*	*aff*	MER	ouloph		COI;ITS;28S	HQ203297,HQ203380,HQ203498
		Oulophyllia	*bennettae*	MER	ouloph		CYTB;28S	AB117358,HQ203496,HQ203497
		Oulophyllia	*crispa*	MER	ouloph		CYTB;28S	AB117356,AB117357,HQ203499
		Oulophyllia	*sp*	MER	ouloph		12S	EF597004
	Paramonstastraea	*Favites*	*peresi*	MER	paramo	*	COI;18S	HE648514,HE648515,HE648516,HE654598,HE654599
		Montastraea	*salebrosa*	MER	paramo	*	COI;18S;28S	HQ203290,HQ203373,HQ203488
	Pectinia	*Pectinia*	*alcicornis*	MER	pectin		CYTB;18S;28S	AB117364,HQ203382,HQ203500
		Pectinia	*lactuca*	MER	pectin		COI;ITS;18S;28S	HQ203300,HQ203384,HQ203384,HQ203502
		Pectinia	*paeonia*	MER	pectin		CYTB;COI;28S	AB117365,HQ203301,HQ203503
	undefined genus	*Platygyra*	*cf*	MER	platxxx	*	COI;ITS;28S	EU371722,HQ203394,HQ203515
		Platygyra	*sp*	MER	platxxx		ITS;16S;18S	AB214159,AB214160,AF265611
	Platygyra	*Platygyra*	*acuta*	MER	platyg		18S;28S	HQ203386,HQ203504
		Platygyra	*carnosus*	MER	platyg		CYTB;12S;16S;COI	JX911333
		Platygyra	*contorta*	MER	platyg		COI;28S	HQ203505,JN248783

Family	Genus	*Genus*	*Species*	Fam. code	Gen. code	*	Genes	No. GenBank Access
		Platygyra	*daedalea*	MER	platyg		CYTB;COI;ITS;12S;16S;18S;28S	AB117281,AB117362,AF549231,HE648532,HE648532,HE648533,HE648534,HE648535,HE654616,HE654617,HE654618.HQ203388.HQ203506.HQ203507.JQ347822.JQ347844
		Platygyra	*lamellina*	MER	platyg		CYTB;COI;18S;28S	AB117282,AB117363,FJ345441,HQ203302,HQ203389,HQ203508,HQ203509
		Platygyra	*pini*	MER	platyg		COI;ITS;18S;28S	HQ203303,HQ203390,HQ203510,HQ203511
		Platygyra	*ryukyuensis*	MER	platyg		COI;28S	HQ203304,HQ203512
		Platygyra	*sinensis*	MER	platyg		COI;ITS;12S;18S;28S	AF177047,AF263361,AF481885,AF481886,AF481887,AF481888,AF481889,AF481890,AF481891,AF481892,AF481895,AF481896,AF481897,AF481898,AF481900,AF481901,HQ203305,HQ203393,HQ203393,HQ203513.HO203514
	Scapophyllia	*Scapophyllia*	*cylindrica*	MER	scapop		CYTB;COI;ITS;18S;28S	AB441198,AB441283,FJ345444,HQ203395,HQ203516,U65479
	Trachyphyllia	*Trachyphyllia*	*geoffroyi*	MER	trachy		CYTB;COI;12S;18S;28S	AB117287,AB117372,HQ203306,HQ203396,HQ203517
Micrabacidae	*Letepsammia*	*Letepsammia*	*formosissima*	MIC	leteps		COI;12S;16S;28S	GQ868662,GQ868663,GQ868664,GQ868665,GQ868668,GQ868669,GQ868670,GQ868672,GQ868684,GQ868685,GQ868691,GQ868692,GQ868696,GQ868697
		Letepsammia	*sp*	MIC	leteps		16S	GQ868688
	Rhombopsammia	*Rhombopsammia*	*niphada*	MIC	rhombo		COI;12S;16S;28S	GQ868661,GQ868674,GQ868683,GQ868693,GQ868695
	Stephanophyllia	*Stephanophyllia*	*complicata*	MIC	stephy		16S;28S	GQ868689,HQ439608
Montastreidae	*Montastraea*	*Montastraea*	*cavernosa*	MON	montas		CYTB;COI;ITS;12S;18S;28S	AB117288,AB117289,AB117373,AB117374,AY451353,AY451354,EF597006,EF597007,EU262810,EU262822,HM447251,HM447252,HM447253,HM447254,HM447255,HM447256,HM447257,HM447258,HM447259,HM447263,HM447264,HM447265,HM447266,HM447267,HM447268,HM447269,HM447270,HM447271,HM447272,HM447273,HM447275,HM447276,HM447278,HM447280,HM447281,HM447283,HM447284,HM447290,HM447293,HM447294,HM447295,HM447296,HM447297,HM447298,HM447299,HM447300,HM447301,HM447303,HM447304,HM447307,HM447309,HM447310,HM447313,HM447314,HM447316,HM447317,HM447318,HM447320,HM447321,HM447331,HM447333,HM447334,HM447336,HM447338,HM447339,HM447340,HM447341,HM447342,HM447345,HM447346,HQ203283,HQ203368,HQ203480,KJ573680,KJ573681
		Montastrea	*cavernosa*	MON	montas		COI	AF013736,AF051094,AF108710,AF108711,AF108712,AF108713,AF108714,AF108715
Mussidae	*Colpophyllia*	*Colpophyllia*	*natans*	MUS	colpop		CYTB;12S;16S; COI	AB117228,AB117306,AY451346,AY451347,DQ643833,KJ573656
	Diploria	*Diploria*	*clivosa*	MUS	diplor		COI;12S;28S	AB117226,EF597001,EU262804
		Diploria	*labyrinthiformis*	MUS	diplor		CYTB;COI;12S;28S	AB117224,AB117302,AY451348,EF597002,EU262772
		Diploria	*strigosa*	MUS	diplor		CYTB;COI;12S	AB117225,AB117303,AF108716,AF108717,AY451349,EF597003,JX183696,KJ573665,KJ573666
	Favia	*Favia*	*fragum*	MUS	favia		CYTB;COI;12S;16S;18S;28S	AB117222,AB117223,AB117300,AB117301,AF549222,AY451350,AY451351,EF597005,EU262856,GQ152753,GQ152792,GQ152793,GQ152813,GQ152857,GQ152870,KJ573668,KJ573669.U40295
		Favia	*leptophylla*	MUS	favia		CYTB;COI	AB117230,AB117307
	Isophyllia	*Isophyllia*	*sinuosa*	MUS	isophy	*	CYTB;COI	AB117238,KJ573674,KJ573675
	Manicina	*Manicina*	*areolata*	MUS	manici		CYTB;12S;28S	AB117305,AF549255,EF597012
	Mussa	*Mussa*	*angulosa*	MUS	mussa		CYTB;COI;12S;16S,18S;28S	AB117316,AB441402,AF549236,AY451363,DQ643834,DQ643834,DQ643834,DQ643834,EF597011,EU262869
	Mussismilia	*Mussismilia*	*harttii*	MUS	mussis		CYTB	AB117308
		Mussismilia	*hispida*	MUS	mussis		COI	FJ216364
	Mycetophyllia	*Mycetophyllia*	*aliciae*	MUS	myceto		CYTB;COI;12S;28S	AB117312,AY451364,EF597039,EU262809
		Mycetophyllia	*daniana*	MUS	myceto		CYTB;COI	AB117234,AB117311
		Mycetophyllia	*lamarckiana*	MUS	myceto		12S;28S	AF549238,EF597040,EU262780
		Mycetophyllia	*sp*	MUS	myceto		CYTB;12S;28S	EF597014,EU262838,KJ573688,KJ573689
	Scolymia	*Scolymia*	*vitiensis*	MUS	parasc		COI	HM048840
	Pseudodiploria	*Pseudodiploria*	*strigosa*	MUS	psedip		COI	KF579902
		Scolymia	*cubensis*	MUS	scolym		CYTB	AB117313
		Scolymia	*sp*	MUS	scolym		CYTB;COI	AB117248,AB117325
Oculinidae	*Cyathelia*	*Cyathelia*	*axillaris*	OCU	cyathe		COI	HM018622
	Madrepora	*Madrepora*	*oculata*	OCU	madrep		CYTB;COI;ITS;12S;16S;28S	HM018659,HQ439680,JQ611302,JQ611395,JQ611434,JX236041
		Oculina	*diffusa*	OCU	oculin		CYTB;COI;ITS;18S;28S	AB117293,AB117379,AB441404,AF549240,FJ966871
		Oculina	*patagonica*	OCU	oculin		12S;16S;28S	AF265601,EF597025,EU262842
		Oculina	*robusta*	OCU	oculin		COI	FJ966869,FJ966874
		Oculina	*sp*	OCU	oculin		COI	AY451365
		Oculina	*varicosa*	OCU	oculin		COI	FJ966867,FJ966868,FJ966872,FJ966873,FJ966875
	Pocillopora	*Pocillopora*	*damicornis*	POC	pocill		CYTB;COI;12S;16S;28S	AF333043,AY139813,EF526302,EU262867,EU400213,HQ420826,L76019

Family	Genus	*Genus*	*Species*	Fam. code	Gen. code	*	Genes	No. GenBank Access
		Pocillopora	*environmental*	POC	pocill		COI	JF905640,JF905691
		Pocillopora	*eydouxi*	POC	pocill	*	CYTB;COI;12S;16S	EF526303
		Pocillopora	*meandrina*	POC	pocill		12S;16S;28S	EF596976,EF596977,EU262803,L76018
		Pocillopora	*verrucosa*	POC	pocill		CYTB;COI;28S	AB441230,AB441315,AF549252,AY139812
		Pocillopora	*woodjonesi*	POC	pocill		COI	KC706677
	Seriatopora	*Seriatopora*	*caliendrum*	POC	seriat		CYTB;COI;12S;16S	EF633524,EF633525,EF633526,EF633527,EF633528,EF6336012
		Seriatopora	*hystrix*	POC	seriat		CYTB;COI;12S;16S	AB441234,AB441319,EF633514,EF633515,EF633516,EF633517,EF633518,EF633519,EF633520,EF633521,EF633522.EF633523.EF633529.EF633530.EF633531.EF6336002.KJ573694.KJ573695
		Seriatopora	*sp*	POC	seriat		CYTB;COI	AB441232,AB441233,AB441317,AB441318
	Stylophora	*Stylophora*	*pistillata*	POC	stylop		CYTB;COI;12S;16S;28S	AB441231,AB441316,AF333044,AF549253,EF633532,EU400214,FR819681,KJ573704,KJ573705
Poritidae	*Bernardpora*	*Goniopora*	*stutchburyi*	POR	bernar	*	COI;ITS;28S	AB907002,AB907004,AB907005,AB907006,AB907011,AB907012,AB907014,AB907016,AB907061,AB907064.JO966133
	undefined genus	*Goniopora*	*cf*	POR	gnpxxx	*	COI;18S	AB748686,AB748687,AB748688,AB748689,AB748690,AB748691,AB748692,AB748694,AB748713,AB748715,AB748716,AB748717,AB748718,AB748719,AB748720,AB748721,AB748722,AB748724,AB748725,AB748726,AB748728,AB748729,AB748731,AB748732,AB748736,AB748737,AB748738,AB748739,AB748740,AB748751,AB748753,AB748769,AB748785,AB748793,AB906947,AB906948,AB906949,AB906950,AB906951,AB906952,AB906958,AB906959,AB906960,AB906961,AB906971,AB906974,AB907030
	undefined genus	*Goniopora*	*sp*	POR	gnpxxx	*	CYTB;COI;12S;16S;28S;	AB441241,AB441326,FJ423995,JQ347813,JQ347836,JQ966144,L76007
	Goniopora	*Goniopora*	*albiconus*	POR	goniop		COI;18S	AB906942,AB906943,AB906944,AB906945,AB907026,AB907027
		Goniopora	*burgosi*	POR	goniop		COI	AB907028
		Goniopora	*ciliatus*	POR	goniop		18S	AB906953
		Goniopora	*columna*	POR	goniop		CYTB;COI;12S;16S;18S	AB441414,AB907031,JF825141,JQ347812,JQ347835,JQ911530
		Goniopora	*djiboutiensis*	POR	goniop		COI;18S	AB748697,AB748700,AB748701,AB748702,AB748704,AB748705,AB748706,AB748707,AB748708,AB748709,AB748710,AB748711,AB748712,AB748775,AB748777,AB906955,AB906956,AB907033
		Goniopora	*gracilis*	POR	goniop		ITS;12S;28S	JQ911529,JQ911536,JQ966130
		Goniopora	*lobata*	POR	goniop		COI;18S	AB748741,AB748742,AB748743,AB748744,AB748745,AB748747,AB748749,AB748750,AB748788,AB748789,AB748790,AB748791,AB906962
		Goniopora	*minor*	POR	goniop	*	COI;18S;12S	AB906964,AB907037,AB907038,JQ911531
		Goniopora	*norfolkensis*	POR	goniop		COI;18S	AB906968,AB906969,AB906970,AB907040,AB907041
		Goniopora	*pendulus*	POR	goniop		COI;18S	AB748676,AB748677,AB748678,AB748679,AB748681,AB748683,AB748685,AB748766
		Goniopora	*planulata*	POR	goniop		12S;16S;18S	JQ347811,JQ347834,JQ911528,JQ911544
		Goniopora	*somaliensis*	POR	goniop		COI;18S	AB906975,AB906976,AB906977,AB906978,AB907047
		Goniopora	*stokesi*	POR	goniop		COI;12S;16S;18S	AB748662,AB748666,AB748667,AB748673,AB748674,AB748675,AB748762,AB906985,AB906986,AB906987.EF597060.L76008
		Goniopora	*tenuidens*	POR	goniop		COI	AB907057,AB907058,AB907059,AB907060
		Poritipora	*paliformis*	POR	goniop	*	COI	AB907043
	Porites	*Porites*	*annae*	POR	porite		COI	FJ423965
		Porites	*astreoides*	POR	porite		CYTB;COI;12S;28S	AB441242,AB441327,AY451374,AY451375,EF597055,EU262821,EU262830,FJ423961,FJ423989
		Porites	*branneri*	POR	porite		COI;12S;28S	AY451380,EF597059,EU262801
		Porites	*colonensis*	POR	porite		COI	FJ423972
		Porites	*compressa*	POR	porite		COI;12S;16S;28S	EF597053,EF597054,EU262784,EU262814,FJ423970,FJ423971,FJ423982,JQ966149,L76020
		Porites	*cylindrica*	POR	porite		COI	FJ423968,FJ423996
		Porites	*divaricata*	POR	porite		COI;12S;28S	AY451381,EF597058,EU262877,FJ423969
		Porites	*duerdeni*	POR	porite		COI	FJ423976,FJ423977
		Porites	*evermanni*	POR	porite		COI	FJ423984,FJ423985
		Porites	*evermanni*	POR	porite		COI	
		Porites	*furcata*	POR	porite		COI	FJ423988
		Porites	*lichen*	POR	porite		COI	FJ423963,FJ423964,FJ423987

Family	Genus	*Genus*	*Species*	Fam. code	Gen. code	*	Genes	No. GenBank Access
		Porites	*lobata*	POR	porite		COI;ITS;16S	AF550372,AY320313,AY320315,AY320322,AY320325,AY320327,AY320331,AY320332,AY320337,AY320339,AY320344,AY320347,AY320348,FJ423973,FJ423974,FJ423975,FJ423983,FJ423994,FN564947,FN564948,FN564950,FN564951,FN564959,FN564964,FN564965,FN564967,FN564968,FN564969,FN564970,FN564971,FN564985,FN564986,FN564988,FN565016,FN565017,FN565018,FN565019,FN565020,FN565028,FN565031,FN565032,FN565036,FN565037,FN565041,FN565042,FN565045,FN565046,FN565048,FN565049,FN565050,FN565052,FN565064,FN565065,FN565067,FN565068,FN565070,FN565071,FN565072,FN565075,FN565089,FN565090,FN565103,FN565106,FN565108,FN565112,FN565113,FN565114,FN565116,FN565120,FN565121,FN565126,FN565127.FN565131.FN565132,FN565133.FN565135.FN565137.FN565138.FN565139
		Porites	*lutea*	POR	porite		CYTB;COI;ITS;12S;28S	AB441243,AB441244,AB441328,AB441329,FJ423967,JQ911533,JQ911583,JQ966142,KF271348,KF271415,KF271418,KF271419.KF271424,KF271436
		Porites	*nigrescens*	POR	porite		12S;18S	JQ911532,JQ911568
		Porites	*okinawensis*	POR	porite		CYTB;COI;12S;16S	JF825142
		Porites	*panamensis*	POR	porite		CYTB;COI;12S;16S	FJ423990,KJ546638
		Porites	*porites*	POR	porite		CYTB;COI;12S;16S;28S	AF549244,DQ643837,EF597056,EF597057,EU262831,EU262878
		Porites	*pukoensis*	POR	porite		12S;18S;28S	JQ911534,JQ911571,JQ966145
		Porites	*randalli*	POR	porite		COI	FJ423966
		Porites	*rus*	POR	porite		COI	FJ423979,FJ423980,FJ423981,FJ423993
		Porites	*solida*	POR	porite		COI	FJ423962,FJ423978
	undefined genus	*Porites*	*cf*	POR	porxxx		COI	AB907073
	undefined genus	*Porites*	*sp*	POR	porxxx		COI;12S;16S	AB907074,FJ423992,JQ347805,JQ347806,JQ347807,JQ347808,JQ347809,JQ347810,JQ347828,JQ347829,JQ347830.JO347831.JO347832.JO347833
	Stylaraea	*Stylaraea*	*punctata*	POR	stylar		COI	AB907066,AB907067,AB907068,AB907069,AB907070,AB907071,AB907072
Psammocoridae	*Psammocora*	*Psammocora*	*albopicta*	PSA	psammo		COI	FM865871,FM865872
		Psammocora	*contigua*	PSA	psammo		CYTB;COI;ITS;18S	AB441209,AB441294,AM494847,AM494848,AM494849,AM494850,AY722782,AY722783,AY722784,HM048842
		Psammocora	*digitata*	PSA	psammo		COI;ITS	AM494854,AM494855,AM494856,AM494857,DQ645394,FM865873,FM865875,FM865876,FM865877
		Psammocora	*haimeana*	PSA	psammo		COI	FM865874
		Psammocora	*nierstraszi*	PSA	psammo		COI	AM494851,AM494852,FM865878
		Psammocora	*profundacella*	PSA	psammo		COI	AM494853,FM865879
		Psammocora	*stellata*	PSA	psammo		16S	L76021
Rhizangidae	*Astrangia*	*Astrangia*	*sp*	RHI	astran		CYTB;12S;16S;COI	DQ643832
Siderastreidae	*Pseudosiderastrea*	*Pseudosiderastrea*	*formosa*	SID	psesid		CYTB;COI	JN600483,JN600484,JN600485
		Pseudosiderastrea	*tayami*	SID	psesid		CYTB;COI;ITS;18S	AM494866,AM494867,AY722789,AY722790,JN600486,JN600487,JN600488
	Siderastrea	*Siderastrea*	*glynni*	SID	sidera		CYTB;ITS	AY322575,AY322576,AY322577,AY322578,AY322579,AY322580,AY322581,AY322582,AY322583,AY322584,AY322585.AY322586.AY322587.AY322588.AY322589,JN600490
		Siderastrea	*radians*	SID	sidera		CYTB;COI;ITS;12S;16S;28S	AB441212,AB441297,AF549246,AY322604,AY322605,AY322606,AY322607,AY322608,AY322609,AY451385,DQ643838,EF597065,EF597066,EU262848,EU262861,JN600493,JN600494,KJ573696,KJ573697
		Siderastrea	*savignyana*	SID	sidera		CYTB;COI	AB441214,AB441215,AB441299,AB441300,AM494868,JN600489,JN600491,JN600492
		Siderastrea	*siderea*	SID	sidera		CYTB;COI;ITS;12S;16S	AB441211,AB441296,AY322590,AY322591,AY322592,AY322593,AY322594,AY322595,AY322596,AY322597,AY322598,AY322599,AY322600,AY322601,AY322602,AY322603,AY451386,AY451387,EF597067,JN600495.JN600496.KJ482944.KJ573698.KJ573699
		Siderastrea	*stellata*	SID	sidera		CYTB;COI;ITS;18S	AB441213,AB441298,AB441407,AY322610,AY322611,AY322612,FJ216363,HM223585,JN600497,JN600498,KF248007
Stenocyathidae	*Stenocyathus*	*Stenocyathus*	*vermiformis*	STE	stenoc		COI;28S	AF549247,HM018619,HQ439681
Turbinoliidae	*Cyathotrochus*	*Cyathotrochus*	*pileus*	TUR	cyatho		COI;16S;28S	HM018623,HQ439682,HQ439764
		Tropidocyathus	*pileus*	TUR	cytaho	*	12S	EF597069
	Notocyathus	*Notocyathus*	*sp*	TUR	notocy		12S;16S;28S	AF265584,EF597061,EU262782
	Tropidocyathus	*Tropidocyathus*	*labidus*	TUR	tropid		12S;16S	AF265585,EF597062
		Tropidocyathus	*lessoni*	TUR	tropid		COI;16S;28S	HM018669,HQ439683,HQ439765
undefined family	undefined genus	*Montastraea*	*sp*	XXX	xxxxxx	*	16S;28S	AF265610,EU262794
undefined family	undefined genus	*Montastrea*	*cf*	XXX	xxxxxx	*	COI	EU371711,EU371712
undefined family	undefined genus	*Favia*	*aff*	favia	favxxx	*	COI;ITS;18S;28S	EU371666,EU371667,FJ345422,EU371707,EU371708,HE648497,HQ203258,HQ203261,HQ203326,HQ203329.HQ203424.HQ203425.HQ203429.HQ203436.HQ203335

Family	Genus	*Genus*	*Species*	Fam. code	Gen. code	*	Genes	No. GenBank Access
undefined family	undefined genus	*Favia*	*sp*	favia	favxxx	*	COI	EU371709,JQ920467
Incertae sedis	*Nemenzophyllia*	*Nemenzophyllia*	*turbida*	INS	nemenz		COI;ITS;18S	HF954193,HF954194,HF954280,HF954281
Incertae sedis	*Plerogyra*	*Plerogyra*	*sinuosa*	INS2	plerog		COI	HE654649,HE654650,HF954196,HF954197
Incertae sedis	undefined genus	*Plerogyra*	*sp*	INS2	plexxx		COI;12S	EF597031,HM018663

Annexe 3. Variability of the protein-coding mitochondrial genes in *D. dianthus* (*Dd*) and *L. pertusa* (*Lp*) individuals. Changes NS= Non-synonymous (**&)** or Synonymous substitutions (S).

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[          1         11        21        31        41        51        61        71        81        91       ]
[          |         |         |         |         |         |         |         |         |         |        ]
Dd636      ATGTATCTTTTAGTTTTGTTTTTCCCTTTAATTGGGGCGGTTTTAGTTGGTTCTTTTGGACGAAAAATAGGGGAAAGGGTGCTGGTGTTTTAACTTCCT
Dd432      ..........................................................................................
Lp_KC875348 ..........................................................A.................................
Lp_KC875349 ..........................................................A.................................
Lp_FR821799 ..........................................................A.................................
Changes NS ..........................................................&.................................

[        101       111       121       131       141       151       161       171       181       191      ]
[          |         |         |         |         |         |         |         |         |         |        ]
Dd636      GTTGTTTAATTATAAGTCTTTCTTATTCTGTTTTTATTGGGGCAGAAATTTTATTTAACTCAACAATAACATATATTAAGTTGTGAAAGTGATTTGATTC
Dd432      ..........C.......................................................................................
Lp_KC875348 ..........C.......................................................................................
Lp_KC875349 ..........C.......................................................................................
Lp_FR821799 ..........C.......................................................................................
Changes NS ..........S.......................................................................................

[        201       211       221       231       241       251       261       271       281       291      ]
[          |         |         |         |         |         |         |         |         |         |        ]
Dd636      AGGATCTTTCCTTGTTTTTTTTTTGGTTTTACAATTTGATTGTTTAGTTGTGATTATGTTGTTTGTTGTTTTTATTGTTTCTACTTTGGTTCATATTTTTTCT
Dd432      ...................................................................................................
Lp_KC875348 ...................................................................................................
Lp_KC875349 ...................................................................................................
Lp_FR821799 ...................................................................................................
Changes NS ...................................................................................................

[        301       311       321       331       341       351       361       371       381       391      ]
[          |         |         |         |         |         |         |         |         |         |        ]
Dd636      ATTGCCTACATGCGAGGAGATCCCCATGTCCCACGGTTTATGTCTTATCTTTCTTTGTTTACTTTTTTTAATGGTTGTTTTTAGTAACAAGTGATAATTTCC
Dd432      .........T.........................................................................................
Lp_KC875348 .........T.........................................................................................
Lp_KC875349 .........T.........................................................................................
Lp_FR821799 .........T.........................................................................................
Changes NS .........S.........................................................................................

[        401       411       421       431       441       451       461       471       481       491      ]
[          |         |         |         |         |         |         |         |         |         |        ]
Dd636      TTCAGTTATTTATTGGCTGGGAGGGGGTAGGCCTTTGTTCTTATTTATTAATTAATTTTTGATTAACAAGATTAGAAGCAAACAGAGCTGCGATTAAAGC
Dd432      ...................................................................G.....................
Lp_KC875348 ...................................................................G.....................
Lp_KC875349 ...................................................................G.....................
Lp_FR821799 ...................................................................G.....................
Changes NS ...................................................................&.....................
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[         501       511       521       531       541       551       561       571       581       591       ]
[                                                                                                             ]
Dd636     AATGTTAGTAAATCGAGTGGGGGACATTGGGTTGCTTTTTAGCAATGTTTCTTATTTGAAATGTTTTTGGGACCTTAGACTTTTCTTCTGTTTTTAATTTA
Dd432     ...................................................................................................
Lp_KC875348 ...................................................................................................
Lp_KC875349 ...................................................................................................
Lp_FR821799 ...................................................................................................
Changes NS ...................................................................................................

[         601       611       621       631       641       651       661       671       681       691       ]
[                                                                                                             ]
Dd636     GTTTTTTGTTGTTCTGATCAAATTTTTTTTATTTGTTTATTCTTGTTCTTAGGAGTGGTTGGTAAATCGGCTCAATTGGGGCTACACACTTGATTACCGG
Dd432     .....................................................G.........................................
Lp_KC875348 .....................................................G.........................................
Lp_KC875349 .....................................................G.........................................
Lp_FR821799 .....................................................G.........................................
Changes NS .....................................................S.........................................

[         701       711       721       731       741       751       761       771       781       791       ]
[                                                                                                             ]
Dd636     ATGCAATGGAAGGTTAGTTGGGCCTTTTAATTAAAAAATTAATTAAAAACGCCACTATGATTATAAAAGCTTTCGTTGTTCTTGTCCCTTTACTTATTGC
Dd432     .....................................................T.........................................
Lp_KC875348 .....................................................T.........................................
Lp_KC875349 .....................................................T.........................................
Lp_FR821799 .....................................................T.........................................
Changes NS .....................................................S.........................................

[         801       811       821       831       841       851       861       871       881       891       ]
[                                                                                                             ]
Dd636     TGTGGCATATTTAACTTTAGCAGAACGAAAGGTTTTAGGGTACATGCAAGCAAGAAAAGGACCTAATGTGGTTGGGGGGGGNTTGCTTCAGCCTTTTGCG
Dd432     .....................................................G.........................................
Lp_KC875348 .....................................................G.........................................
Lp_KC875349 .....................................................G.........................................
Lp_FR821799 .....................................................G.........................................
Changes NS .....................................................S.........................................

[         901       911       921       931       941       951       961       971       981       991       ]
[                                                                                                             ]
Dd636     GATGGGATTAAGTTATTTCTTAAAGAAATGGTTATTCCCCATCGAGTGAGTGGGTTTGTTTATCTTTTAGCCCCAGTTCTTTCTTTTATTTTGGCTTTTA
Dd432     ...................................................................................................
Lp_KC875348 ...................................................................................................
Lp_KC875349 ...................................................................................................
Lp_FR821799 ...................................................................................................
Changes NS ...................................................................................................
```

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[        1001      1011      1021      1031      1041      1051      1061      1071      1081      1091      ]
[        |         |         |         |         |         |         |         |         |         |         ]
Dd636    TTGTTTGGGGTATCCTCCCTTATAATAAGGGGTTGGAATAAGTGATTTTCAAATTGGTCTTTTATGAATCATGGCGATTTCTTCTGTGAGTGTTTATGC
Dd432    ..........................................A.....................................................
Lp_KC875348 ..........T.....................................A.....................................................
Lp_KC875349 ..........T.....................................A.....................................................
Lp_FR821799 ..........T.....................................A.....................................................
Changes NS   ..........S.....................................&.....................................................

[        1101      1111      1121      1131      1141      1151      1161      1171      1181      1191      ]
[        |         |         |         |         |         |         |         |         |         |         ]
Dd636    TATTTTAATGTCGGGCTGAGGCAGTCGTTCTAAATATGCTTTTTTAGGTGCGGTCCGAGCTGCGGCGCAAATGATTAGTTATGAGGTTTCTATTGGTTTA
Dd432    ..........................................................................................
Lp_KC875348 ..........................................................................................
Lp_KC875349 ..........................................................................................
Lp_FR821799 ..........................................................................................
Changes NS   ..........................................................................................

[        1201      1211      1221      1231      1241      1251      1261      1271      1281      1291      ]
[        |         |         |         |         |         |         |         |         |         |         ]
Dd636    ATTATTATTTCTGTTCTTTTATGTGTTGGGTCTTTGTCTTTAAATGAAATTGTATTAACACAAAATGAAATTTGGTTTTTTTTTTCCCTTTTTTCCTGTTG
Dd432    ..........................................................G.......................................
Lp_KC875348 ..........................................................................................
Lp_KC875349 ..........................................................................................
Lp_FR821799 ..........................................................G.......................................
Changes NS   ..........................................................&.......................................

[        1301      1311      1321      1331      1341      1351      1361      1371      1381      1391      ]
[        |         |         |         |         |         |         |         |         |         |         ]
Dd636    TTATAATGTTWCTCGTTTCTATTTTAGCAGAAACAAACCGTGCTCCCTTTGATTTAACARAAGGAGAGTCGGAGCTRGTGTCGGGGTACAACGTAGAGTA
Dd432    ..........T.....................................G.....A.....................................
Lp_KC875348 ..........T.....................................G.....A.....................................
Lp_KC875349 ..........T.....................................G.....A.....................................
Lp_FR821799 ..........T.....................................G.....A.....................................
Changes NS   ..........S.....................................S.....S.....................................

[        1401      1411      1421      1431      1441      1451      1461      1471      1481      1491      ]
[        |         |         |         |         |         |         |         |         |         |         ]
Dd636    CGCTTCGATGTCTTTTGCTCTATTTTTTTCTTGCCGAATATGCTCATATTATATTTATGAGTTGTTTAACTGTTCTTTTATTTTTTGGAGGATGGTTGCTT
Dd432    ..........................................................................................
Lp_KC875348 ..........................................................................................
Lp_KC875349 ..........................................................................................
Lp_FR821799 ..........................................................................................
Changes NS   ..........................................................................................
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[        1501      1511      1521      1531      1541      1551      1561      1571      1581      1591      ]
[        |         |         |         |         |         |         |         |         |         |         ]
Dd636    TTGCCCCTTGGTTTTTAAAACCGTTTTTTATTGTTTTATTTTTTTTTATGGGCACGGGCCTCTTTCCCTAGGGTCCGGTATGATCAATTGATGGYCCTATTAT
Dd432    ..........................................................................................C.........
Lp_KC875348 ..........................................................................................C.........
Lp_KC875349 ..........................................................................................C.........
Lp_FR821799 ..........................................................................................C.........
Changes NS ..........................................................................................S.........

[        1601      1611      1621      1631      1641      1651      1661      1671      1681      1691      ]
[        |         |         |         |         |         |         |         |         |         |         ]
Dd636    GAAAGGGGTATTTACCTTTAAGTTTAGGGATTGTTATTTTTGTTGCCAGTATTTTATTCGGGTTTAATGGTTCTCCTCCG--ATGAGTGGTGCTTATTTT
Dd432    ..........................................................................ATT.....................
Lp_KC875348 ..........................................................................ATT.....................
Lp_KC875349 ..........................................................................ATT.....................
Lp_FR821799 ..........................................................................ATT.....................
Changes NS ..........................................................................--Y.....................

[        1701      1711      1721      1731      1741      1751      1761      1771      1781      1791      ]
[        |         |         |         |         |         |         |         |         |         |         ]
Dd636    TGATCAATTTTAATATTGTGTGATTATTTGGTGTGACGAACTCGGCAGTAATGATGGGCCTTACAGTTATTATTGTTTTATTGTTTTTTAAATGGGGTGGAT
Dd432    ..........................................................................................T.....
Lp_KC875348 ..........................................................................................T.....
Lp_KC875349 ..........................................................................................T.....
Lp_FR821799 ..........................................................................................T.....
Changes NS ..........................................................................................S.....

[        1801      1811      1821      1831      1841      1851      1861      1871      1881      1891      ]
[        |         |         |         |         |         |         |         |         |         |         ]
Dd636    CTCATCCCAAAAAGATGGCAATCTATTTTAGAGTTAACATATAGTCATTTTTATCGTGTTATAGAAGACAATYTGGGAGGGCGAGGGTTGAAGTATTTCT
Dd432    ..........................................T.....................................................
Lp_KC875348 ..........................................T.....................................................
Lp_KC875349 ..........................................T.....................................................
Lp_FR821799 ..........................................T.....................................................
Changes NS ..........................................S.....................................................

[        1901      1911      1921      1931      1941      1951      1961      1971      1981      1991      ]
[        |         |         |         |         |         |         |         |         |         |         ]
Dd636    CTTTTGTTCTCTCTCTTTTTTCTTTGGGGTTTGTTTGAATGTGCTGGGTTTATGCCCATATGTTTTTACTCCAACCGTTCATATTATAGTTACATTGGG
Dd432    ..........N.............................................................................
Lp_KC875348 ..........N.............................................................................
Lp_KC875349 ..........N.............................................................................
Lp_FR821799 ..........N.............................................................................
Changes NS ..........S.....................................................................................
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[        2001      2011      2021      2031      2041      2051      2061      2071      2081      2091      ]
[        |         |         |         |         |         |         |         |         |         |         ]
Dd636    TTTATCTTTTTTCAATAATCATCGGTGTCACTCTTGCTGGTTTTTTGGAGGTTTAAGTGAGATTTTTTTTAGTGTTTTTTATGCCAAGCGGAGCCCCTCTTGGG
Dd432    ..........................................................................................................
Lp_KC875348 ..........................................................................................................
Lp_KC875349 ..........................................................................................................
Lp_FR821799 ..........................................................................................................
Changes NS  ..........................................................................................................

[        2101      2111      2121      2131      2141      2151      2161      2171      2181      2191      ]
[        |         |         |         |         |         |         |         |         |         |         ]
Dd636    CTTGCCCCCTTTGTTAGTTTTTAATTGAAACAGTTAGTTATATCTCACGGGCTATTTCGTTAGGAGTTCGTTTGGCGGCTAATTTATCGGCTGGGCATCTAT
Dd432    ..........................................................................................................
Lp_KC875348 ..........................................................................................................
Lp_KC875349 ..........................................................................................................
Lp_FR821799 ..........................................................................................................
Changes NS  ..........................................................................................................

[        2201      2211      2221      2231      2241      2251      2261      2271      2281      2291      ]
[        |         |         |         |         |         |         |         |         |         |         ]
Dd636    TATTTGCTATTTTTAGCTGGGTTTGGGTTTTAATATGTTAATTGCAAGTGGGCCGGCGGGGGCTATTGCCCTTGTTAATAATGGTTTTTTATAACACTATTAGA
Dd432    ..........................................................................................................
Lp_KC875348 .......G..................................................................................................
Lp_KC875349 .......G..................................................................................................
Lp_FR821799 .......G..................................................................................................
Changes NS  .......S..................................................................................................

[        2301      2311      2321      2331      2341      2351      2361      2371      2381      2391      ]
[        |         |         |         |         |         |         |         |         |         |         ]
Dd636    AGTGGCGGTGGCCGTAATTCAGGCTTATGTTTTTTGTTTATTAACAACTATTTATTTAGCAGACACACTGATTTTACATATGGGTCTTTTTTTGGCTAGTT
Dd432    ..........................................................................................................
Lp_KC875348 ..........................................................................................................
Lp_KC875349 ..........................................................................................................
Lp_FR821799 ..........................................................................................................
Changes NS  ..........................................................................................................

[        2401      2411      2421      2431      2441      2451      2461      2471      2481      2491      ]
[        |         |         |         |         |         |         |         |         |         |         ]
Dd636    CTTTTTTGTTGGGATCATAAGTGTGACGGGGGCTTCAAGAGAAAAAAGGAGTGTGTTAAAAAAACGGGCCTTAGAGTGATCTATGGCTATTTTTTTTAGCT
Dd432    ..........................................................................................................
Lp_KC875348 ..........................................................................................................
Lp_KC875349 ..........................................................................................................
Lp_FR821799 ..........................................................................................................
Changes NS  ..........................................................................................................
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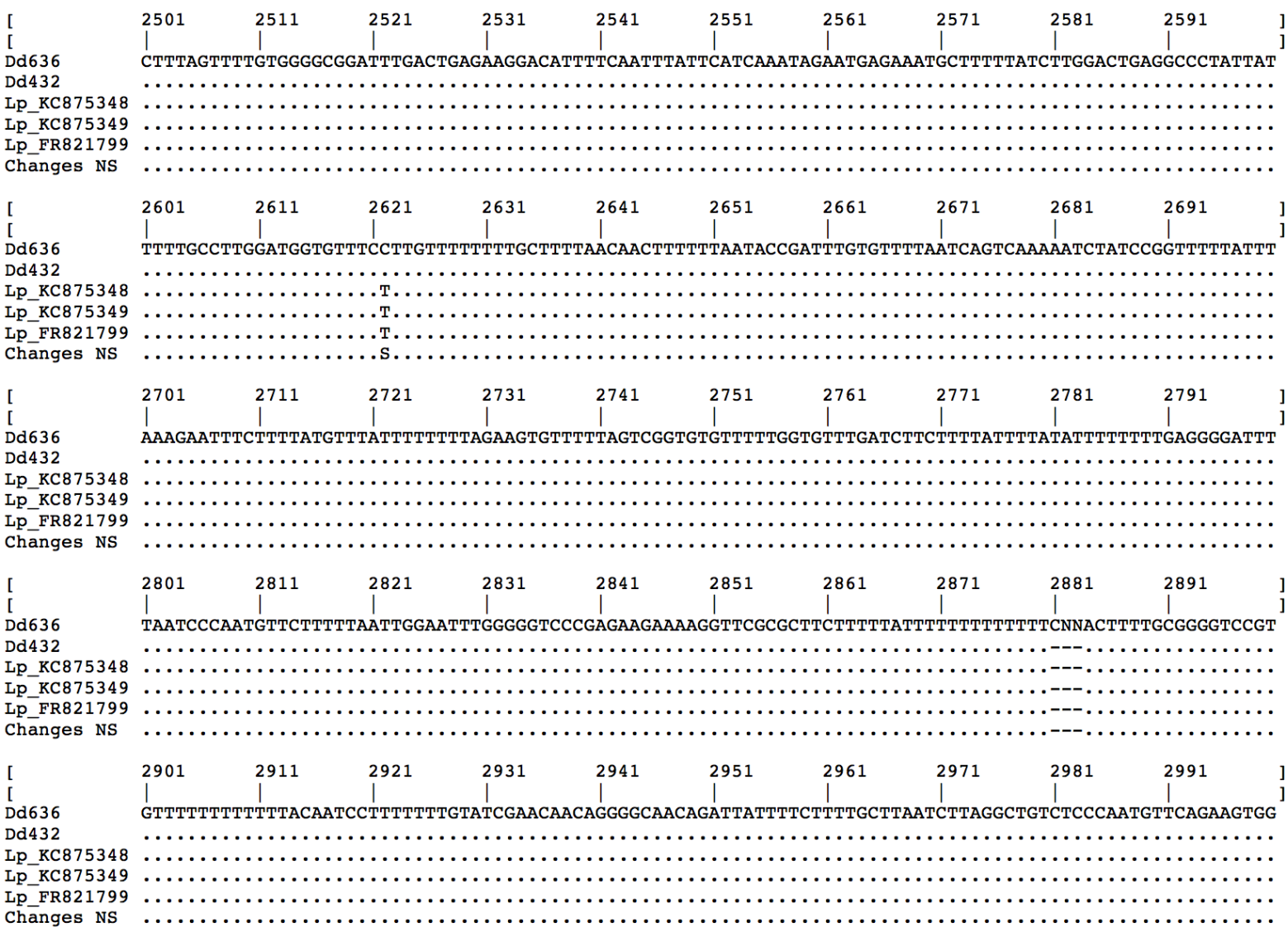
[2501 2511 2521 2531 2541 2551 2561 2571 2581 2591]
Dd636 CTTTAGTTTTGTGGGGCGGATTTGACTGAGAAGGACATTTTCAATTTATTCATCAAATAGAATGAGAAATGCTTTTTATCTTGGACTGAGGCCCTATTAT
Dd432 .....
Lp_KC875348 .....
Lp_KC875349 .....
Lp_FR821799 .....
Changes NS .....
[2601 2611 2621 2631 2641 2651 2661 2671 2681 2691]
Dd636 TTTTGCCTTGGATGGTGTTTCCTTGTTTTTTTTGCTTTTAACAACTTTTTTAATACCGATTTGTGTTTTAATCAGTCAAAAATCTATCCGGTTTTTATTT
Dd432 .....
Lp_KC875348 .....T.....
Lp_KC875349 .....T.....
Lp_FR821799 .....T.....
Changes NS .....S.....
[2701 2711 2721 2731 2741 2751 2761 2771 2781 2791]
Dd636 AAAGAATTTCTTTTATGTTTATTTTTTTTAGAAGTGTTTTTAGTCGGTGTGTTTTTGGTGTTTGATCTTCTTTTATTTTATATTTTTTTTGAGGGGATTT
Dd432 .....
Lp_KC875348 .....
Lp_KC875349 .....
Lp_FR821799 .....
Changes NS .....
[2801 2811 2821 2831 2841 2851 2861 2871 2881 2891]
Dd636 TAATCCCAATGTTCTTTTTAATTGGAATTTGGGGGTCCCGAGAAGAAAAGGTTCGCGCTTCTTTTTATTTTTTTTTTTTTCNNACTTTTGCGGGGTCCGT
Dd432 .....---.....
Lp_KC875348 .....---.....
Lp_KC875349 .....---.....
Lp_FR821799 .....---.....
Changes NS .....---.....
[2901 2911 2921 2931 2941 2951 2961 2971 2981 2991]
Dd636 GTTTTTTTTTTTTACAATCCTTTTTTTGTATCGAACAACAGGGGCAACAGATTATTTTCTTTTGCTTAATCTTAGGCTGTCTCCCAATGTTCAGAAGTGG
Dd432 .....
Lp_KC875348 .....
Lp_KC875349 .....
Lp_FR821799 .....
Changes NS .....

```
[         3001      3011      3021      3031      3041      3051      3061      3071      3081      3091      ]
[         |         |         |         |         |         |         |         |         |         |         ]
Dd636     GCCTTAATTGGCGTCTTTCTTAGTTTTGCAGTTAAAATTCCCTTAATCCCGTTTCATATTTGGCTTCCCCAAGCACATGTGGAAGCCCCTGTTGCGGGCT
Dd432     ..........................................................................................
Lp_KC875348 ..........................................................................................
Lp_KC875349 ..........................................................................................
Lp_FR821799 ..........................................................................................
Changes NS  ..........................................................................................

[         3101      3111      3121      3131      3141      3151      3161      3171      3181      3191      ]
[         |         |         |         |         |         |         |         |         |         |         ]
Dd636     CAGTTATTTTAGCTGGGATTTTATTAAAACTAGGGGGGTATGGGGTTTTACGGTTTTCTTGGCCTCTCTTCCCTGCGGCTTCTGAATATTGATCTCCTGT
Dd432     ..........................................................C...............................
Lp_KC875348 ..........................................................................................
Lp_KC875349 ..........................................................................................
Lp_FR821799 ..........................................................................................
Changes NS  ..........................................................................................

[         3201      3211      3221      3231      3241      3251      3261      3271      3281      3291      ]
[         |         |         |         |         |         |         |         |         |         |         ]
Dd636     TATTGTTTTTTTTTTTCTGWTTTGGCYGTTGTTTATGGGGGGTTTAATGACATGTCGTCAGGTTGACTTTAAACGACTAGTTGCTTATTCTTCGGTGGCACAC
Dd432     ..................T.....T.....................................................................
Lp_KC875348 ..................T.....T.....................................................................
Lp_KC875349 ..................T.....T.....................................................................
Lp_FR821799 ..................T.....T.....................................................................
Changes NS  ..................S.....S.....................................................................

[         3301      3311      3321      3331      3341      3351      3361      3371      3381      3391      ]
[         |         |         |         |         |         |         |         |         |         |         ]
Dd636     ATGGGTTTAGTTCCTCTAGGGGTTTTTACACATATTATAGAAGGGCTTGTTGGAGCTGTTTTTTTAATGTTTAGCCCATGGTTTTGTTAGTTCCGCTCTTT
Dd432     .....CC....................................................................................
Lp_KC875348 .....C....................................................................................
Lp_KC875349 .....C....................................................................................
Lp_FR821799 .....C....................................................................................
Changes NS  .....SS...................................................................................

[         3401      3411      3421      3431      3441      3451      3461      3471      3481      3491      ]
[         |         |         |         |         |         |         |         |         |         |         ]
Dd636     TTATTGGGATTACGTATTTATATGATCGCCATCATACTCGTTTAATTAAATATATTATCGTGGCTTGGCTTTAACGATGCCGCTTTTTTGCTATTTTCAATGTT
Dd432     ..........................................................................................
Lp_KC875348 ..........................................................................................
Lp_KC875349 ..........................................................................................
Lp_FR821799 ..........................................................................................
Changes NS  ..........................................................................................
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[                3501      3511      3521      3531      3541      3551      3561      3571      3581      3591      ]
[                                                                                                                  ]
Dd636            AATTTTTGTCCTTAACAAACATGGGCTTCCCGTTAAGTAGTAATTTTGTTGGAGAGTTTTTTTCTTTGTTAGCAGCTTTTTAAGTATCATTTGGGGGTTGGG
Dd432            ..........................................................Y.....................................
Lp_KC875348      ..........................................................................................
Lp_KC875349      ..........................................................................................
Lp_FR821799      ..........................................................................................
Changes NS       ..........................S...............................................................

[                3601      3611      3621      3631      3641      3651      3661      3671      3681      3691      ]
[                                                                                                                  ]
Dd636            GGTTTTTGTTGTTTTTAGGAGTTATTTTTTTCTGTTGTTTATTCTCTTAGTTTGTTTAWTCGGATTTTCTTTTTGGGGGYGGTTCTAATTATCTTCTTTTTAACA
Dd432            ..........................................A.....................C.....................................
Lp_KC875348      ..........................................A.....................C.....................................
Lp_KC875349      ..........................................A.....................C.....................................
Lp_FR821799      ..........................................A.....................C.....................................
Changes NS       ..........................S...............................S.....................................

[                3701      3711      3721      3731      3741      3751      3761      3771      3781      3791      ]
[                                                                                                                  ]
Dd636            GAGATTTAAGTCGACAAGAAGCTTTTGTCATGCTTCCTTTTCTTGTAATTATTTTTTTTTGGGGGCGTTGTCCCTTTTTTTTATTCTTGATTTAATAAGAAA
Dd432            ..........................................................................................
Lp_KC875348      ..........................................................................................
Lp_KC875349      ..........................................................................................
Lp_FR821799      ..........................................................................................
Changes NS       ..........................................................................................

[                3801      3811      3821      3831      3841      3851      3861      3871      3881      3891      ]
[                                                                                                                  ]
Dd636            TTGTCTTGTGTTTTAGTCCGATTGGATATGACTATCATCTTGTGGAGTTTTCTCCTTGACCTTTTATTGGAGCTGCCGGGGCCTTCTTCTTGACTGTGGGG
Dd432            ..........................................................................................
Lp_KC875348      ..........................................................................................
Lp_KC875349      ..........................................................................................
Lp_FR821799      ..........................................................................................
Changes NS       ..........................................................................................

[                3901      3911      3921      3931      3941      3951      3961      3971      3981      3991      ]
[                                                                                                                  ]
Dd636            GCAGTTGTTTTTTTTTCATTATGGTTTGACTTTTTTTTTTTGGGTTTAGGGGGCGCTGATTGTACTTGGGGTGATGTTTGTTTGATGACAAGACATTATACGAG
Dd432            ..........................................................................................
Lp_KC875348      ..........................................................................................
Lp_KC875349      ..........................................................................................
Lp_FR821799      ..........................................................................................
Changes NS       ..........................................................................................
```

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[        4001      4011      4021      4031      4041      4051      4061      4071      4081      4091      ]
[                                                                                                          ]
Dd636    AAGCGACTTTTTCAGGGACACCATTCTTTAGTTGTAAAGCAAGGCCTTAAGTATGGCATGCTTTTGTTTATTCTTTCAGAAGTTTTGTTTTTTTTTTCTTT
Dd432    ..........................................................................................................
Lp_KC875348 ..........................................................................................................
Lp_KC875349 ..........................................................................................................
Lp_FR821799 ..........................................................................................................
Changes NS ..........................................................................................................

[        4101      4111      4121      4131      4141      4151      4161      4171      4181      4191      ]
[                                                                                                          ]
Dd636    TTTTTGAGCTTTTTTTTCATAGTAGTTTGGCTCCGGCTATTGAACTTGGGGTTGTGTGACCACCGCAAGGTGTTCATGCGCTAAATCCTTTTTTCTGTTCCA
Dd432    ..........................................................................................................
Lp_KC875348 ..........................................................................................................
Lp_KC875349 ..........................................................................................................
Lp_FR821799 ..........................................................................................................
Changes NS ..........................................................................................................

[        4201      4211      4221      4231      4241      4251      4261      4271      4281      4291      ]
[                                                                                                          ]
Dd636    TTGTTAAGCACGGCCGTTTTTATTGAGTTCTGGGGCATCGGTAACGTGGGCCCATCATGCTATAATAAGTGGGAATAAGAAAGAAGCGGTTGCAGGTTTGT
Dd432    .......A..................G.....................................................................
Lp_KC875348 .......A..........................................................................................
Lp_KC875349 .......A..........................................................................................
Lp_FR821799 .......A..........................................................................................
Changes NS .......&..........................................................................................

[        4301      4311      4321      4331      4341      4351      4361      4371      4381      4391      ]
[                                                                                                          ]
Dd636    CTTTGACTATTTTATTGGGTGTGGTGTTTACAGGTTTACAGGCCCTTGAATATTATGAAGCTCCCTTTGCTCTTTCCGATTCTGTTTATGGCTCCACTTT
Dd432    ..........................................................C.......................................
Lp_KC875348 ..........................................................................................................
Lp_KC875349 ..........................................................................................................
Lp_FR821799 ..........................................................................................................
Changes NS ..........................S.....................................................................

[        4401      4411      4421      4431      4441      4451      4461      4471      4481      4491      ]
[                                                                                                          ]
Dd636    TTTTGTTGCAACAGGGTTTCATGGGTTACACGTGATAATTGGAACAACTTTTTTGTTTGTTTGTTTTCTTCGGTTACTTTCCAATCAGTTTACCCGTAGC
Dd432    ..........................................................................................................
Lp_KC875348 ..........................................................................................G..
Lp_KC875349 ..........................................................................................G..
Lp_FR821799 ..........................................................................................G..
Changes NS ..........................................................................................&..
```

```
[          4501      4511      4521      4531      4541      4551      4561      4571      4581      4591      ]
[          |         |         |         |         |         |         |         |         |         |         ]
Dd636      CAACATCTGGGTTTTGAAGCGGCAAGTTGATATTGGCATTTTGTCGATGTTGTTTGGTTGTTTTTATATCTTTCAATTTATTTGTGGGGTTCTAGAGATG
Dd432      ..........................................................................................
Lp_KC875348 .....................C....................................................................
Lp_KC875349 ....................C.....................................................................
Lp_FR821799 ....................C.....................................................................
Changes NS ....................S.....................................................................

[          4601      4611      4621      4631      4641      4651      4661      4671      4681      4691      ]
[          |         |         |         |         |         |         |         |         |         |         ]
Dd636      AGCCAGAGGCTTGGGTTTTGGGGTTTCAGGATGTGGCAGACCCGGTAGTAGAAGAAATTGTTTTTTTTTCATGATCAAGTAATGTTTTTATTAATCATTAT
Dd432      ..........................................................................................
Lp_KC875348 ..........................................................................................
Lp_KC875349 ..........................................................................................
Lp_FR821799 ..........................................................................................
Changes NS ..........................................................................................

[          4701      4711      4721      4731      4741      4751      4761      4771      4781      4791      ]
[          |         |         |         |         |         |         |         |         |         |         ]
Dd636      TGTCACTGTTGTTTTATGGCTTATTGTGGAAGCTTTTTGAAATAAATTTTATGATCGTAATTTAGTTGATGGTACTTTTTTTAGAAATTGTTTGAACAATA
Dd432      ..........................................................................................
Lp_KC875348 ..........................................................................................
Lp_KC875349 ..........................................................................................
Lp_FR821799 ..........................................................................................
Changes NS ..........................................................................................

[          4801      4811      4821      4831      4841      4851      4861      4871      4881      4891      ]
[          |         |         |         |         |         |         |         |         |         |         ]
Dd636      ATCCCCCGCTGTTATATTGATTTTTATTGCACTACCCTCGTTAAAATTGTTGTATTTAATGGACGAAATCATTTCTCCTGCTTTAACAATTAAAGTCATTG
Dd432      ..........................................................................................
Lp_KC875348 ..........................................................................................
Lp_KC875349 ..........................................................................................
Lp_FR821799 ..........................................................................................
Changes NS ..........................................................................................

[          4901      4911      4921      4931      4941      4951      4961      4971      4981      4991      ]
[          |         |         |         |         |         |         |         |         |         |         ]
Dd636      GCCATCAATGATATTGGTCTTATGAATACTCTGATTTATGAAGGCGAGACGTTAGCTTTTGATTCTTATATGGTTCCGTCTTCGGATTTAATTTCAGGGAA
Dd432      ..........................................................................................
Lp_KC875348 ..........................................................................................
Lp_KC875349 ..........................................................................................
Lp_FR821799 ..........................................................................................
Changes NS ..........................................................................................
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[                5001       5011       5021       5031       5041       5051       5061       5071       5081       5091       ]
[                |          |          |          |          |          |          |          |          |          |          ]
Dd636            AAACCGTTTACTTGAAGTGGATCAAAAACTTGTTGTTCCAATTGGAACTCATATAAGATTTTTAGTGACGGGAGCCGATGTCTTGCATTCTTTTGCGGTC
Dd432            ..........................................G.....................................................
Lp_KC875348      ..........................................................................................
Lp_KC875349      ..........................................................................................
Lp_FR821799      ..........................................................................................
Changes NS       ..........................................&.....................................................

[                5101       5111       5121       5131       5141       5151       5161       5171       5181       5191       ]
[                |          |          |          |          |          |          |          |          |          |          ]
Dd636            CCTTCTTTAGGATTAAAAGTAGACGCTGTGCCTGGCCGTTTAAATCAAACTGGTGTTTTTATCAAACGACCGGGGGTTTTTTTT---GGGCAATGCTCTG
Dd432            ..........................................................................TNN.............
Lp_KC875348      ..........................................................................---.............
Lp_KC875349      ..........................................................................---.............
Lp_FR821799      ..........................................................................---.............
Changes NS       ..........................................................................---.............

[                5201       5211       5221       5231       5241       5251       5261       5271       5281       5291       ]
[                |          |          |          |          |          |          |          |          |          |          ]
Dd636            AGATTTGTGGGGCGAATCACTCTTTTTATGCCTATTGTTATAGAGGGAGTCGGGTTAAATGAATATATTATGTATTATAAGTATTTAGTTGTTGTCATGGT
Dd432            ..........................................................................................
Lp_KC875348      ..........................................................................................
Lp_KC875349      ..........................................................................--.....................
Lp_FR821799      ..........................................................................---.....................
Changes NS       ..........................................................................---.....................

[                5301       5311       5321       5331       5341       5351       5361       5371       5381       5391       ]
[                |          |          |          |          |          |          |          |          |          |          ]
Dd636            TTTATTTTTATTAGGAGGTTGGGGGGGTAGTTTTAAATCGAGGACATTTTATTATAATGATTGTTTCAATTGAACTTGTTTTATTAGCAACTTTTTTTTTTT
Dd432            ..........................................................................................
Lp_KC875348      ..........................................................................................
Lp_KC875349      ..........................................................................................
Lp_FR821799      ..........................................................................................
Changes NS       ..........................................................................................

[                5401       5411       5421       5431       5441       5451       5461       5471       5481       5491       ]
[                |          |          |          |          |          |          |          |          |          |          ]
Dd636            TTTTTTNNGATAAATTCTAAGGAAATAGACGCTTTAATAGAACAGGTTTTTATGATAATGGGTTTGACAATTGCGGCGGCAGAGTCTTCTATTGGCTTGG
Dd432            ....N---.....................Y.....................................................................
Lp_KC875348      .....---.....................................................................................
Lp_KC875349      .....---.....................................................................................
Lp_FR821799      .....---.....................................................................................
Changes NS       ....S---.....................S.....................................................................
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[        5501      5511      5521      5531      5541      5551      5561      5571      5581      5591      ]
[                                                                                                          ]
Dd636    CTCTTTTAGTGGCCTATTACCGAATTAGGGGAACGATTGTTTTAAAATCCTTTAGTTCTTTACGGGGTTTTTTTTTTTTGTTTTAATAGCAGTTGTCTTAGC
Dd432    ..........................................................................................................
Lp_KC875348 ..........................................................................................................
Lp_KC875349 ..........................................................................................................
Lp_FR821799 ..........................................................................................................
Changes NS  ..........................................................................................................

[        5601      5611      5621      5631      5641      5651      5661      5671      5681      5691      ]
[                                                                                                          ]
Dd636    CGGGGTATTTTTCTAGTATTTCTTTTTTTTATTGGAGAAAAAAGACCGGACCGAGAAAAAGTGTCTGCTTATGAGTGTGGGTTTGTACCTTTTAGTTTTCTG
Dd432    ..........................................................................................................
Lp_KC875348 ..........................................................................................................
Lp_KC875349 ..........................................................................................................
Lp_FR821799 ..........................................................................................................
Changes NS  ..........................................................................................................

[        5701      5711      5721      5731      5741      5751      5761      5771      5781      5791      ]
[                                                                                                          ]
Dd636    GGGCGCCCCCTTTTCTGTGCGGTTCTTTTTTAATTGGTATTTTTGTTCTTAATTTTTTGACTTAGAAATTTCTTTTTTTATTTCCTTGGTGTGTTTTTATATAATT
Dd432    ..........................................................................................................
Lp_KC875348 ..........................................................................................................
Lp_KC875349 ..........................................................................................................
Lp_FR821799 ..........................................................................................................
Changes NS  ..........................................................................................................

[        5801      5811      5821      5831      5841      5851      5861      5871      5881      5891      ]
[                                                                                                          ]
Dd636    CACTTGGACCTTTTGGGTTTTGAGCCATGGTTGGGTTTTTGTTTATATTAACTGTTGGCCTGGTTTATGAGTGACTAAAAGGAGGGCTAGAATGGGAGCCC
Dd432    ..........................................................................................................
Lp_KC875348 ..........................................................................................................
Lp_KC875349 ..........................................................................................................
Lp_FR821799 ..........................................................................................................
Changes NS  ..........................................................................................................

[        5901      5911      5921      5931      5941      5951      5961      5971      5981      5991      ]
[                                                                                                          ]
Dd636    AACTCCGGTTTCTGCCTTAATCCATGCGGCAACAATGGTTACGGCAGGTGTTTTTTTGTTAATTAGAGCTTCCCCTCTTTTTGATGTYGTTCCTTTTATG
Dd432    ..........................................................................................................
Lp_KC875348 ..........................................................................C...........................
Lp_KC875349 ..........................................................................C...........................
Lp_FR821799 ..........................................................................C...........................
Changes NS  ..........................................................................S...........................
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[         6001      6011      6021      6031      6041      6051      6061      6071      6081      6091      ]
[         |         |         |         |         |         |         |         |         |         |         ]
Dd636     TTGGTTTTTATAACGATAATAGGGGTGTTAACAGTTTTTGTTGCGGGAACAATTGGTCTTGTTCAAAATGATTTAAAAAAAATAATTGCTTATTCCACTT
Dd432     ..........................................................................................
Lp_KC875348 ..........................................................................................
Lp_KC875349 ..........................................................................................
Lp_FR821799 ..........................................................................................
Changes NS  ..........................................................................................

[         6101      6111      6121      6131      6141      6151      6161      6171      6181      6191      ]
[         |         |         |         |         |         |         |         |         |         |         ]
Dd636     GTAGTCAATTGGGGTATATGGTTGTGGCTTGTTGGTCTTTCTCATTTTTCTATTGGTCTTTTCCACTTAATGAATCATGCTTTTTTTTAAGGCTTTGTTATT
Dd432     ..........................................................................................
Lp_KC875348 ..........................................................................................
Lp_KC875349 ..........................................................................................
Lp_FR821799 ..........................................................................................
Changes NS  ..........................................................................................

[         6201      6211      6221      6231      6241      6251      6261      6271      6281      6291      ]
[         |         |         |         |         |         |         |         |         |         |         ]
Dd636     TTTAAGTGCTGGTTCTTTAATTCATGCAATGATAGACGAACAAGACATAAGAAAAATGGGGGGCTTATTACAAATCAYACCTTTGACTTATATTTTTTTTT
Dd432     ..........................................................T.......................................
Lp_KC875348 ..........................................................T.......................................
Lp_KC875349 ..........................................................T.......................................
Lp_FR821799 ..........................................................T.......................................
Changes NS  ..........................................................S.......................................

[         6301      6311      6321      6331      6341      6351      6361      6371      6381      6391      ]
[         |         |         |         |         |         |         |         |         |         |         ]
Dd636     ATTATAGGCTCTTTTTCTTTAATGGGATTTCCTTTTTTTAACCGGGTTTTATTCAAAAGACTTAATCTTAGRAGTTACTTTTGGGCAATATTATTTAATTT
Dd432     .....................................A....................................................
Lp_KC875348 .....................................A....................................................
Lp_KC875349 .....................................A....................................................
Lp_FR821799 .....................................A....................................................
Changes NS  .....................................S....................................................

[         6401      6411      6421      6431      6441      6451      6461      6471      6481      6491      ]
[         |         |         |         |         |         |         |         |         |         |         ]
Dd636     TTGTTTATTGGCTAGGTTGTTTTTCTGTTTTTATTAACAGCAATGTATTCAATCCGTTTGGTTYATTATGCCTTTTTCTCTAATACAAATTCTAAAAGGGC
Dd432     ..........................T.......................................................
Lp_KC875348 ..........................T.......................................................
Lp_KC875349 ..........................T.......................................................
Lp_FR821799 ..........................T.......................................................
Changes NS  ..........................S.......................................................
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[          6501      6511      6521      6531      6541      6551      6561      6571      6581      6591      ]
[          |         |         |         |         |         |         |         |         |         |         ]
Dd636      GGTTTTTTGCCTCGTTAAAAGAGGAGAGGGCCTTTGTTGGCCCCTTTGGGGGTTTTAGCCTTAGGTAGTCTTTTTTTGGGGTTATTTATGTAAAGAAATT
Dd432      ..........................................................................................
Lp_KC875348 ..........................................................................................
Lp_KC875349 ..........................................................................................
Lp_FR821799 ..........................................................................................
Changes NS ..........................................................................................

[          6601      6611      6621      6631      6641      6651      6661      6671      6681      6691      ]
[          |         |         |         |         |         |         |         |         |         |         ]
Dd636      GTGTGGTCTTTTTCAAATGGGGCTTTCGCCAATGCTTTTTTTTAAAATAAAAATGCTTCCTGTTATAGTAAGTATTATGGGGCATTGGGGGTTATTTTTG
Dd432      ..........................................................................................
Lp_KC875348 ..........................................................................................
Lp_KC875349 ..........................................................................................
Lp_FR821799 ..........................................................................................
Changes NS ..........................................................................................

[          6701      6711      6721      6731      6741      6751      6761      6771      6781      6791      ]
[          |         |         |         |         |         |         |         |         |         |         ]
Dd636      TTTTATTTAATTTTTTCTTCTAAAATATTGTTTTTTTTTGTTTTCTCTTTATACCTTTTTTGGCTCTGCTTGACAAATAAATTCTTTTTTTTAATTTTTTTT
Dd432      ..........C...........................................................................
Lp_KC875348 ..........................................................................................
Lp_KC875349 ..........................................................................................
Lp_FR821799 ..........................................................................................
Changes NS ..........S...............................................................................

[          6801      6811      6821      6831      6841      6851      6861      6871      6881      6891      ]
[          |         |         |         |         |         |         |         |         |         |         ]
Dd636      TATAAAAAAAAATCTATAAAACGGGGCATCTGATTATGAATTTAACTGTTGACAAAGGTGTCTTAGAGCTGGTTGGGCCCAGGGGCCTTGTTCAATTTCTA
Dd432      ..........................................................................................
Lp_KC875348 ..........................................................................................
Lp_KC875349 ..........................................................................A...............
Lp_FR821799 ..........................................................................A...............
Changes NS ..........................................................&...............................

[          6901      6911      6921      6931      6941      6951      6961      6971      6981      6991      ]
[          |         |         |         |         |         |         |         |         |         |         ]
Dd636      GTCCGCCAAATTCAAAGACTTAGTGATTTACAGTCGGGGCAAGTTTTTAATTATGCTTTAGTTATACTAATTGGTGTTGTCATATTTATTTGGGGAAAAG
Dd432      ..........................................................................................
Lp_KC875348 ..........................................................................................
Lp_KC875349 ..........................................................................................
Lp_FR821799 ..........................................................................................
Changes NS ..........................................................................................
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[        7001      7011      7021      7031      7041      7051      7061      7071      7081      7091      ]
[        |         |         |         |         |         |         |         |         |         |         ]
Dd636    AAAACCCTCTTTTGTCTCTGGTCAATGGATTCTTAGTGAATTTGCCCTCTCCCTCCAACATAAGTTATTTTTGAAATTTTGGGTCTTTATTGGGCCTGTG
Dd432    ..........................................................................................
Lp_KC875348 ..........................................................................................
Lp_KC875349 ..........................................................................................
Lp_FR821799 ..........................................................................................
Changes NS ..........................................................................................

[        7101      7111      7121      7131      7141      7151      7161      7171      7181      7191      ]
[        |         |         |         |         |         |         |         |         |         |         ]
Dd636    TTTGGTGCTACAAATTATAACAGGCTGTTTTTTGTCTATGCATTATTGTCCAGATGTTAATTTAGCTTTTGCTTCTATCGGCCATATTATGCGGGATGTG
Dd432    ..........................................................................................
Lp_KC875348 ..........................................................................................
Lp_KC875349 ..........................................................................................
Lp_FR821799 ..........................................................................................
Changes NS ..........................................................................................

[        7201      7211      7221      7231      7241      7251      7261      7271      7281      7291      ]
[        |         |         |         |         |         |         |         |         |         |         ]
Dd636    AACTCTGGTTTTTTTATTAAAATATTTACATGCAAATGGTGCTGCTTTATTTTTTTTTTTGTCTTTATGTTCATGTTGGGCGGGGCTTGTATTATGGGAGTT
Dd432    ..........................................T...............................................
Lp_KC875348 ..........................................................................................
Lp_KC875349 ..........................................................................................
Lp_FR821799 ..........................................................................................
Changes NS ..........................................S...............................................

[        7301      7311      7321      7331      7341      7351      7361      7371      7381      7391      ]
[        |         |         |         |         |         |         |         |         |         |         ]
Dd636    ATTTGAAATTTCATGTTTGAAGTGTTGGGGTTGTAATTTTTTTATTAACGATGGCGACGGCTTTTATGGGTTATGTTTTGCCTTGAGGGCAAATGTCTTT
Dd432    ..........................................................................................
Lp_KC875348 ..........................................................................................
Lp_KC875349 ..........................................................................................
Lp_FR821799 ..........................................................................................
Changes NS ..........................................................................................

[        7401      7411      7421      7431      7441      7451      7461      7471      7481      7491      ]
[        |         |         |         |         |         |         |         |         |         |         ]
Dd636    TTGAGGAGCCACTGTTATCACAAATTTATTGTCTGCTATTCCTTACTTTGGGGTAGACATTGTTCAATGGGTTTGAGGGGGTTTTAGTGTTTCTGGGGCA
Dd432    ..........................................................................................
Lp_KC875348 ..........................................................................................
Lp_KC875349 ..........................................................................................
Lp_FR821799 ..........C.................................................................................
Changes NS ..........&.....................................................................................
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[          7501       7511       7521       7531       7541       7551       7561       7571       7581       7591       ]
[          |          |          |          |          |          |          |          |          |          |          ]
Dd636      ACCTTAAATCGGTTTTTTAGTTTACATTTTTTACTTCCTTTTTTTTTTGGTTGTTTTTTGTTTTTATTCATTTAATGTATTTACATGTTGATGGGTCAAATA
Dd432      ..........................................................................................................
Lp_KC875348 ..........................................................................................................
Lp_KC875349 ..........................................................................................................
Lp_FR821799 ..........................................................................................................
Changes NS ..........................................................................................................

[          7601       7611       7621       7631       7641       7651       7661       7671       7681       7691       ]
[          |          |          |          |          |          |          |          |          |          |          ]
Dd636      ACCCGACGGGGTTRAACTCTTCTAATGAGAATGTTTCTTTCCATACTTTTTTATACTTCAAAAGATCTTTTTGGGTTTTTTTTCTTTTTATTTATTTTTG
Dd432      ..........A...............................................................................................
Lp_KC875348 ..........A...............................................................................................
Lp_KC875349 ..........A...............................................................................................
Lp_FR821799 ..........A...............................................................................................
Changes NS ..........S...............................................................................................

[          7701       7711       7721       7731       7741       7751       7761       7771       7781       7791       ]
[          |          |          |          |          |          |          |          |          |          |          ]
Dd636      TTTGTTTGTTTTTTTTTTGACCTAATTTGTTGGGGGACTCGGAAARCTTTATTCAAGCGAACTCTTTGGTCACTCCTGTGCACATTCAGCCAGAATGGTAT
Dd432      ..........................................A....R.....................................................
Lp_KC875348 ..........................................A.....................................................
Lp_KC875349 ..........................................A.....................................................
Lp_FR821799 ..........................................A.....................................................
Changes NS ..........................................S....S.....................................................

[          7801       7811       7821       7831       7841       7851       7861       7871       7881       7891       ]
[          |          |          |          |          |          |          |          |          |          |          ]
Dd636      TTTTTATTTGCTTATGCAATCTTGCGTTCAATACCCAATAAGTTGGGGGGGGTCATTGCAATGTTTTTGTAGCATTTTAGTTTTGTTTTTACTACCAATTT
Dd432      ..........................................................................T...........................
Lp_KC875348 ..........................................................................T...........................
Lp_KC875349 ..........................................................................T...........................
Lp_FR821799 ..........................................................................T...........................
Changes NS ..........................................................................S...........................

[          7901       7911       7921       7931       7941       7951       7961       7971       7981       7991       ]
[          |          |          |          |          |          |          |          |          |          |          ]
Dd636      TACACAAAAGTGTACTAAAGGGGTGTTCTTTTCGCCCTCTTGGGCGTGTCGCCTTTTGATTTTTTGCTTGTTGATTTCGGCCTTTTAACTTGAATTGGGGC
Dd432      ..........................................................................................Y...........
Lp_KC875348 ..........................................................................................Y...........
Lp_KC875349 ..........................................................................................Y...........
Lp_FR821799 ..........................................................................................Y...........
Changes NS ..........................................................................................S...........
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[                8501       8511       8521       8531       8541       8551       8561       8571       8581       8591       ]
[                |          |          |          |          |          |          |          |          |          |          ]
Dd636            TATTGGCCCACTGTGCCGAAGATAGGGATTTTYTCTCTTTTAATTGCGCTCGGTTTGCCAGTTAATTCTTTATTAATTGGGGTTGTTTTTATCTTTATTTGT
Dd432            ..........................T..........................G..........Y..........................T..........
Lp_KC875348      ..........................T..........................G..........................T..........
Lp_KC875349      ..........................T..........................G..........................T..........
Lp_FR821799      ..........................T..........................G..........................T..........
Changes NS       ..........................S..........................S..........................&..........

[                8601       8611       8621       8631       8641       8651       8661       8671       8681       8691       ]
[                |          |          |          |          |          |          |          |          |          |          ]
Dd636            TGGAACTTTAGGGGCCTTAAACCAAACAAAAATTAAACGACTATTGGCCTATAGTAGTATTGGTCATATGGGCTTTATTCTATGGGGCTTCGAGAGTGGT
Dd432            .......K..................................................................................................
Lp_KC875348      ..........................................................................................................
Lp_KC875349      ..........................................................................................................
Lp_FR821799      ..........................................................................................................
Changes NS       .......S..................................................................................................

[                8701       8711       8721       8731       8741       8751       8761       8771       8781       8791       ]
[                |          |          |          |          |          |          |          |          |          |          ]
Dd636            TCCTTTGAAAGTTTACAAGCCAGTTTGGTCYATCTTTTYATATATGTTATTATTATGACTATCTGTGTTTTTTCTCTTATATTGGGCTTTCGTTTATATAAGA
Dd432            ..........................T..........T..........................................A..........
Lp_KC875348      ..........................T..........T..........................................A..........
Lp_KC875349      ..........................T..........T..........................................A..........
Lp_FR821799      ..........................T..........T..........................................A..........
Changes NS       ..........................S..........S..........................................&..........

[                8801       8811       8821       8831       8841       8851       8861       8871       8881       8891       ]
[                |          |          |          |          |          |          |          |          |          |          ]
Dd636            ATTTACTTATATAGAATTTAGTGGGGTGTCTCGATTTTTTACCTCTTTTTTGCCGTTACTTTAGGCGTCGTATTTTTTTTCTATTGCTGGAATTCCTCCTTTTGC
Dd432            ..........................................................................................................
Lp_KC875348      ..........................................................................C...........................
Lp_KC875349      ..........................................................................C...........................
Lp_FR821799      ..........................................................................C...........................
Changes NS       ..........................................................................S...........................

[                8901       8911       8921       8931       8941       8951       8961       8971       8981       8991       ]
[                |          |          |          |          |          |          |          |          |          |          ]
Dd636            AGGATTTTTAAGTAAATGAGTTGTTTTTGTTGTCTGGAGTACTTTCTCAGTCTTATTTTGTTTTTCTATTTGCCGTTTTTTTGTTCTGTAATAGGGGGTGTT
Dd432            ..........................................................................................A.......
Lp_KC875348      ..........................................................................................A.......
Lp_KC875349      ..........................................................................................A.......
Lp_FR821799      ..........................................................................................A.......
Changes NS       ..........................................................................................S.......
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[          9001      9011      9021      9031      9041      9051      9061      9071      9081      9091      ]
[          |         |         |         |         |         |         |         |         |         |         ]
Dd636      TATTATGTTAGAATTGTCAAAATACTTTTTTTTTCAAAAAAACTCTTATCTTTAATTACGATAAAAGCTCTAAGAAAAGAATTACACTTAAATTTTAAAA
Dd432      ..........................................................................................
Lp_KC875348 ..........................................................................................
Lp_KC875349 ..........................................................................................
Lp_FR821799 ..........................................................................................
Changes NS ..........................................................................................

[          9101      9111      9121      9131      9141      9151      9161      9171      9181      9191      ]
[          |         |         |         |         |         |         |         |         |         |         ]
Dd636      AAGTCTTTTTGGTTGGTCTTTGTCTTTATTTTATTCTGTTTCTTTTTTGTGGCTCCTCATTTAGGTTTTTTTTTTGCTTCTCAAACAATAATGATATTGTT
Dd432      ..........................................................................................
Lp_KC875348 ..........................................................................................
Lp_KC875349 ..........................................................................................
Lp_FR821799 ..........................................C.................................................
Changes NS ..........................................&.................................................

[          9201      9211      9221      9231      9241      9251      9261      9271      9281      9291      ]
[          |         |         |         |         |         |         |         |         |         |         ]
Dd636      TTTTGGGGCAATTGGTTCTGGGATAATGGTTATTTCTGCATTAAACCCTGTATTTTTCTCTTTTTTGATTGGTTATCGTTTTTTATTAATTCTGCAGTTTTT
Dd432      ..........................Y.......................................................Y.....
Lp_KC875348 ..........................................................................................
Lp_KC875349 ..........................................................................................
Lp_FR821799 ..........................................C.......................................C.....
Changes NS ..........................S.....................................&.....................S.....

[          9301      9311      9321      9331      9341      9351      9361      9371      9381      9391      ]
[          |         |         |         |         |         |         |         |         |         |         ]
Dd636      TTTCTTTTGTTTAGGAGTTGATTTTTATTGCTTTTAATGTTTTTTGTTGGTTTTATGTTGGGGCCATTGCTGTTTTTATTTTTTGTTTGTTATTATGTTGTTTAAATT
Dd432      ..........................................................................................
Lp_KC875348 ..........................................................................................
Lp_KC875349 ..........................................................................................
Lp_FR821799 ..........................................................................................
Changes NS ..........................................................................................

[          9401      9411      9421      9431      9441      9451      9461      9471      9481      9491      ]
[          |         |         |         |         |         |         |         |         |         |         ]
Dd636      TAACGGATTTATCCTCCTGCTTTTTTTTTAAAAGAGAGGCCGACATGACAAATTATATTCCGATTGGGTTCCTTATAGGTGTCTTTTTTTTTTTCTGAGGCCTC
Dd432      ..........C...............................................................................
Lp_KC875348 ..........................................................................................
Lp_KC875349 ..........................................................................................
Lp_FR821799 ..........................................................................................
Changes NS ..........S.................................................................................
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```
[          9501      9511      9521      9531      9541      9551      9561      9571      9581      9591      ]
[          |         |         |         |         |         |         |         |         |         |         ]
Dd636      TTCTAGTTGATTCGTTTTGGGACCTTTCCAAAAAGAGAACTGAGACTTGTCTTTTCCTTGACTTATTATTTCTTATCATAATATAGAGGCGCTGGGGCAA
Dd432      ..........T.....................................................................................
Lp_KC875348 ..........T.....................................C.................................................
Lp_KC875349 ..........T.....................................................................................
Lp_FR821799 ..........T.....................................................................................
Changes NS ..........S.....................................&...............................................

[          9601      9611      9621      9631      9641      9651      9661      9671      9681      9691      ]
[          |         |         |         |         |         |         |         |         |         |         ]
Dd636      GTTTTATATGTTGTTTGTTGTTTATTTATTTTGGCCAGCTTTATTCTTTTAGTTGCTATGCTCGGAGCTATTTTTTTTAACTCAAGATATGATGATAAATA
Dd432      ..Y.....................................................................C.....................
Lp_KC875348 ..........................................A..........................................A.....
Lp_KC875349 ..........................................A..........................................A.....
Lp_FR821799 ..........................................A..........................................A.....
Changes NS ..S.....................................&...............................................S.....

[          9701      9711      9721      9731      9741      9751      9761      9771      9781      9791      ]
[          |         |         |         |         |         |         |         |         |         |         ]
Dd636      AATATTTAATTCGTTGAGTTTTTTCTACAAACCACACAAAGATATCGGTACTTTATATTTAGTTTTTGGGGTTGGGGCGGGTTTAATTGGAACGGCTTTTAG
Dd432      ..........................................................................................
Lp_KC875348 ..........................................................................................
Lp_KC875349 ..........................................................................................
Lp_FR821799 ..........................................................................................
Changes NS ..........................................................................................

[          9801      9811      9821      9831      9841      9851      9861      9871      9881      9891      ]
[          |         |         |         |         |         |         |         |         |         |         ]
Dd636      TATGCTTATACGACTGGAGCTTTCTGCGCCGGGGGCGATGCTGGGGGACGATCATCTTTATAAATGTCATTGTAACAGCACATGCTTTTTATTATGATTTTT
Dd432      ..........................................................................................
Lp_KC875348 ..........................................................................................
Lp_KC875349 ..........................................................................................
Lp_FR821799 ..........................................................................................
Changes NS ..........................................................................................

[          9901      9911      9921      9931      9941      9951      9961      9971      9981      9991      ]
[          |         |         |         |         |         |         |         |         |         |         ]
Dd636      TTTTTAGTTATGCCCGTTATGATTGGTGGGTTYGGTAATTGGTTGGTTCCACTATATATTGGAGCCCCTGATATGGCTTTCCCCCGATTAAACAATATTA
Dd432      ..........T.....................................................................................
Lp_KC875348 ..........T.....................................................................................
Lp_KC875349 ..........T.....................................................................................
Lp_FR821799 ..........T.....................................................................................
Changes NS ..........S.....................................................................................
```

```
[          10001     10011     10021     10031     10041     10051     10061     10071     10081     10091     ]
[          |         |         |         |         |         |         |         |         |         |         ]
Dd636      GTTTTTGGTTGTTGCCGCCGGCATTGTTTTTATTATTGGGTTCGGCTTTTGTAGAACAAGGAGTAGGAACAGGGTGAACGGTTTATCCCCCGTTGTCCAG
Dd432      ..........................................................................................
Lp_KC875348 ..........................................T.....................C.....................................
Lp_KC875349 ..........................................T.....................C.....................................
Lp_FR821799 ..........................................T.....................C.....................................
Changes NS ..........................................&.....................&.....................................

[          10101     10111     10121     10131     10141     10151     10161     10171     10181     10191     ]
[          |         |         |         |         |         |         |         |         |         |         ]
Dd636      TGTCCAAGCACACTCCGGGGGTTCTGTTGACATGGCGATTTTTAGTCTCCATTTGGCTGGGGCTTCTTCTATTTTAGGGGCAATAAACTTTATTACTACA
Dd432      ..........................................................................................
Lp_KC875348 ..........................................................................................
Lp_KC875349 ..........................................................................................
Lp_FR821799 ..........................................T.....................T.....................................
Changes NS ..........................................&.....................&.....................................

[          10201     10211     10221     10231     10241     10251     10261     10271     10281     10291     ]
[          |         |         |         |         |         |         |         |         |         |         ]
Dd636      ATTTTTAATATGCGGGCCCCGGGTATTACGTTTAATAAAATGCCTTTGTTTGTTTGGTCTATTTTAATCACTGCTTTTTTATTGCTTTTATCTTTACCTG
Dd432      ..........................................................................................
Lp_KC875348 ..........................................................................................
Lp_KC875349 ..........................................................................................
Lp_FR821799 ..........................................................................................
Changes NS ..........................................................................................

[          10301     10311     10321     10331     10341     10351     10361     10371     10381     10391     ]
[          |         |         |         |         |         |         |         |         |         |         ]
Dd636      TTTTAGCCGGTGCTATTACAATGCTTTTTAACAGATCGTAATTTTTAACACCACTTTTTTTGAGCCTTCAGGGGGGGGGGATCCGATTTTATTTTCAGCATTT
Dd432      ..........................................................................................
Lp_KC875348 ..........................................................................................
Lp_KC875349 ..........................................................................................
Lp_FR821799 ..........................................................................................
Changes NS ..........................................................................................

[          10401     10411     10421     10431     10441     10451     10461     10471     10481     10491     ]
[          |         |         |         |         |         |         |         |         |         |         ]
Dd636      ATTTTTGGTTTTTTTGGACACCCAGAAGTTTATATTTTTGATTTTTACCGGGCTTTGGGATGATCTCTCTCAAATTATTCCTACTTTTGTTGCAAAAAAACAAATT
Dd432      ..........................................................................................
Lp_KC875348 ..........................................................................................
Lp_KC875349 ..........................................................................................
Lp_FR821799 ..........................................................................................
Changes NS ..........................................................................................
```

```
[           10501     10511     10521     10531     10541     10551     10561     10571     10581     10591     ]
[           |         |         |         |         |         |         |         |         |         |         ]
Dd636       TTTGGATATTTAGGAATGGTTTATGCAATGCTTTCTATTGGGCTTCTTGGGTTTATTGTATGGGCACATCATATGTTTACTGTTGGTATGGATGTTGATA
Dd432       ..........................................................................................
Lp_KC875348 ..........................................................................................
Lp_KC875349 ..........................................................................................
Lp_FR821799 ..........................................................................................
Changes NS  ..........................................................................................

[           10601     10611     10621     10631     10641     10651     10661     10671     10681     10691     ]
[           |         |         |         |         |         |         |         |         |         |         ]
Dd636       CAAGGGCATATTTTACAGCAGCAACGATGATAATTGCTGTTCCTACGGGGATTAAGGTTTTTAGTTGATTAGCTACTGTTTACGGGGGGGTTTTAAGATT
Dd432       ..........................................................................................
Lp_KC875348 ..........................................................................................
Lp_KC875349 ..........................................................................................
Lp_FR821799 ..........................................................................................
Changes NS  ..........................................................................................

[           10701     10711     10721     10731     10741     10751     10761     10771     10781     10791     ]
[           |         |         |         |         |         |         |         |         |         |         ]
Dd636       AGACACTCCAATGCTTTGGGCTATGGGATTTGTTTTTTTATTTACATTAGGCGGGCTTACTGGGGTAATTTTAGCCAATAGTTCTCTTGATATTGTTCTT
Dd432       ..........................................................................................
Lp_KC875348 ..........................................................................................
Lp_KC875349 ..........................................................................................
Lp_FR821799 ..........................................................................................
Changes NS  ..........................................................................................

[           10801     10811     10821     10831     10841     10851     10861     10871     10881     10891     ]
[           |         |         |         |         |         |         |         |         |         |         ]
Dd636       CATGATACATATTATGTTGTAGCACATTTTCATTATGTCCTTTCTATGGGGGCCGTGTTTGCTATCTTTGGGGGGTTTTATTATTGAATCGGAAAAATTA
Dd432       ..........................................................................................
Lp_KC875348 ..........................................................................................
Lp_KC875349 ..........................................................................................
Lp_FR821799 ..........................................................................................
Changes NS  ..........................................................................................

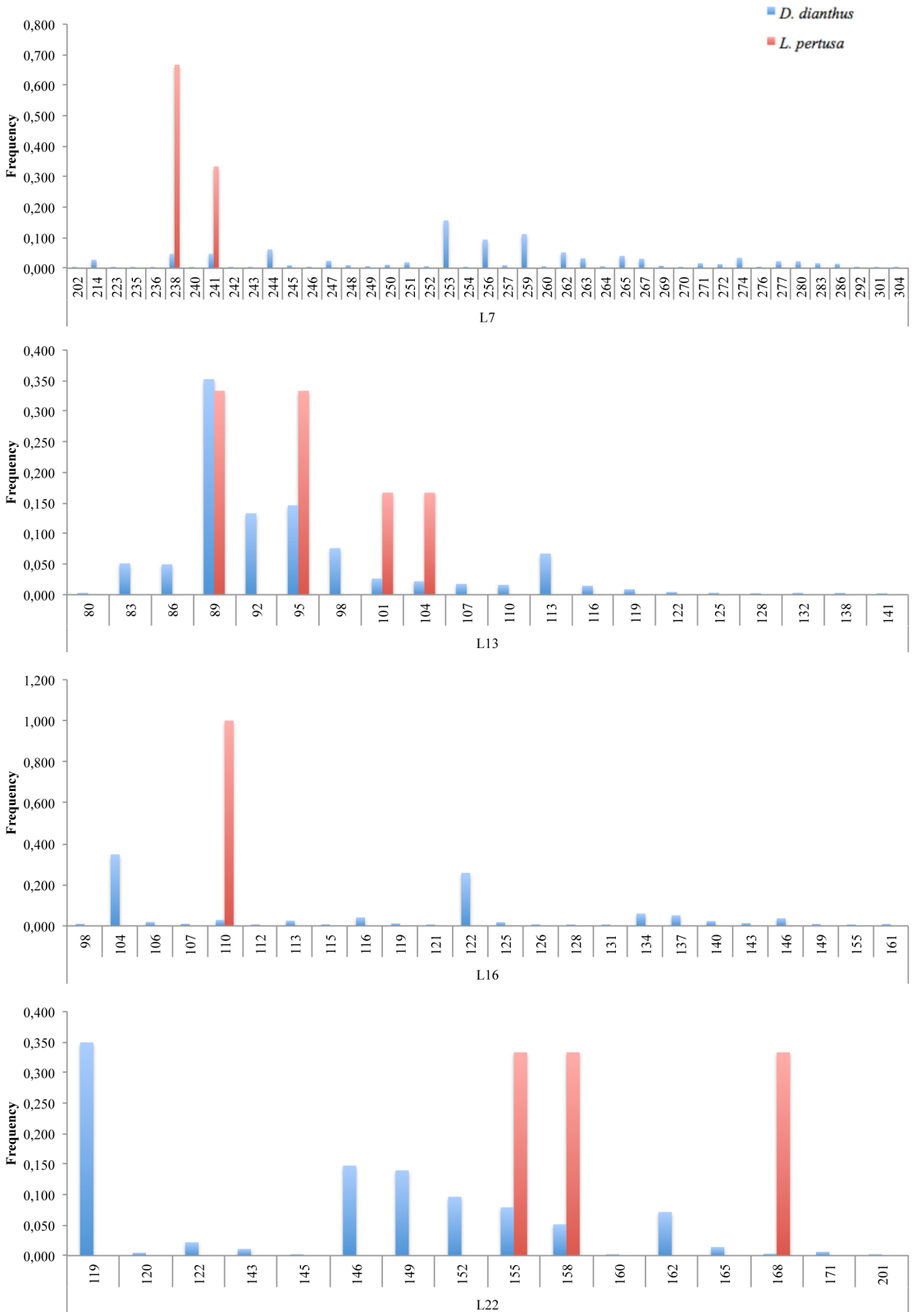
[           10901     10911     10921     10931     10941     10951     10961     10971     10981     10991     ]
[           |         |         |         |         |         |         |         |         |         |         ]
Dd636       GTGGTTATTGTTATAATGAGCTTTTTGGAAAAGTTCATTTTTGGTTGATGTTTATCGGAGTAAATTTAACTTTTTTCCCTCAACATTTTTTAGGTTTAAC
Dd432       ..........................................................................................
Lp_KC875348 ..........................................................................................
Lp_KC875349 ..........................................................................................
Lp_FR821799 ..........................................................................................
Changes NS  ..........................................................................................
```

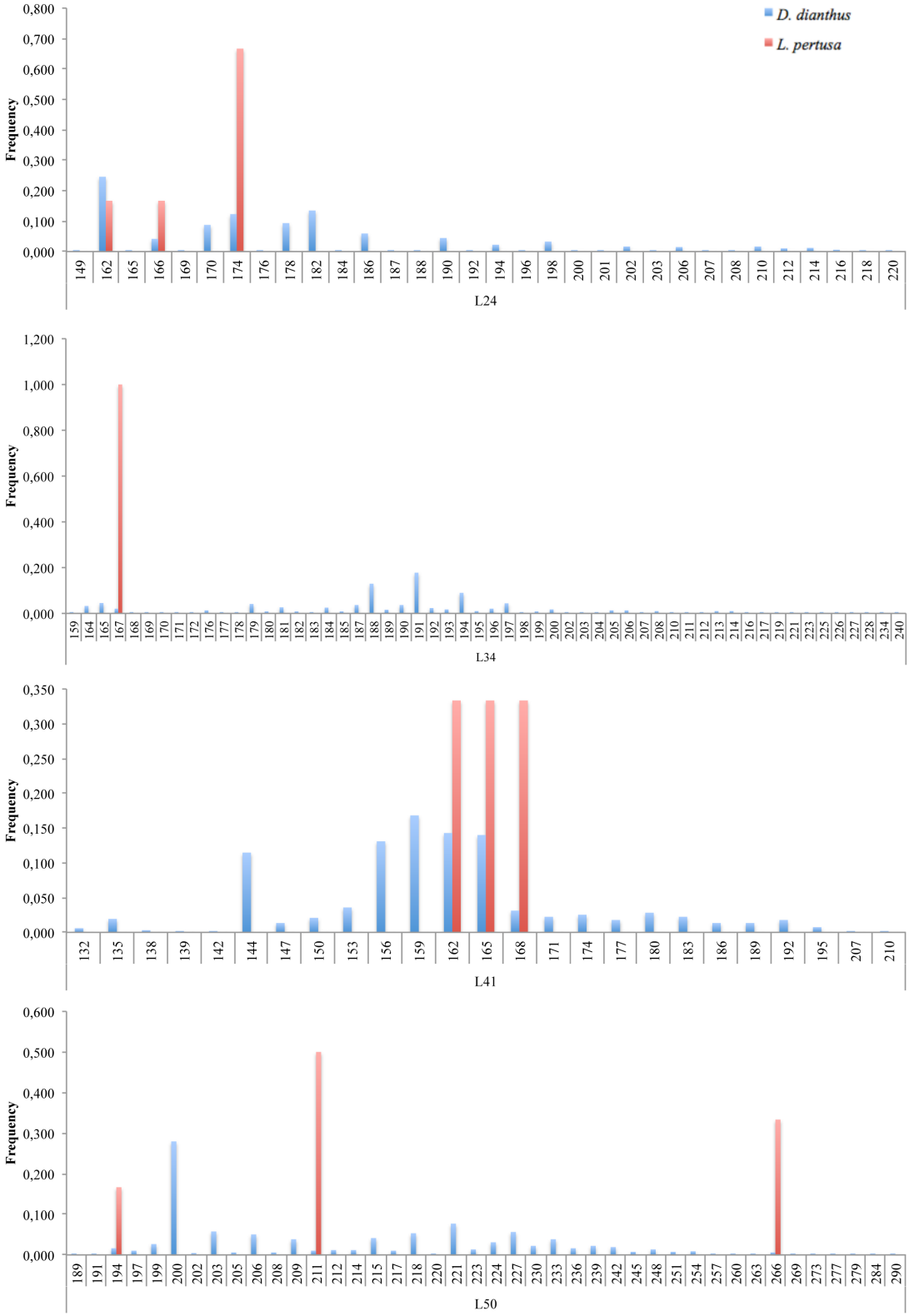
```
[          11001     11011     11021     11031     11041     11051     11061     11071     11081     11091     ]
[          |         |         |         |         |         |         |         |         |         |         ]
Dd636      GGGGTTCCCCAGACGATATTCGGATTTTTGCAGATTCTTTTTGCTGGTTGGAACCTAATTAGTTCTTTTGGTTCTGTTATTTCTATTTTTAGGTGTTATATGA
Dd432      ..........................................................................................
Lp_KC875348 ..........................................................................................
Lp_KC875349 ..........................................................................................
Lp_FR821799 ..........................................................................................
Changes NS ..........................................................................................

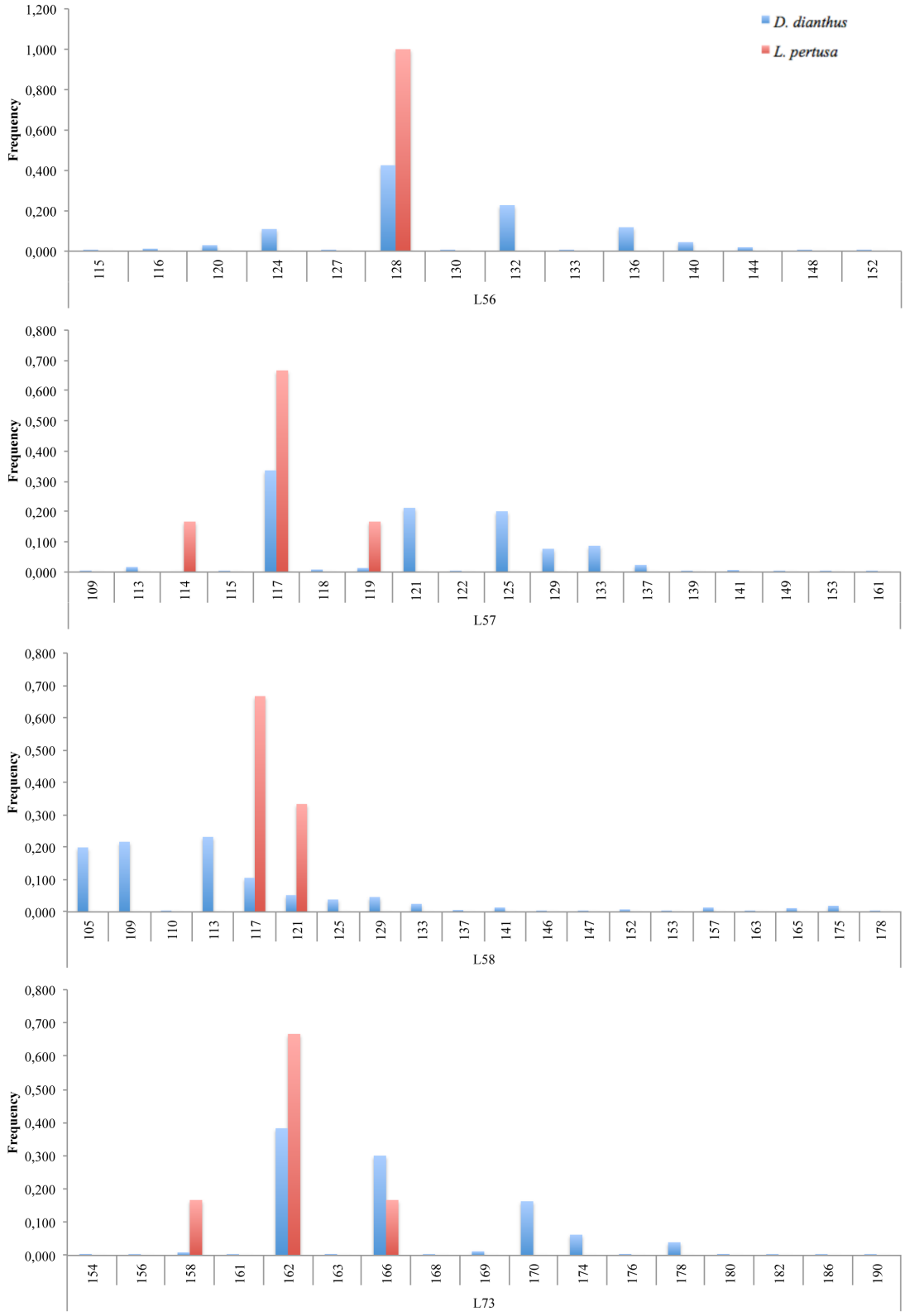
[          11101     11111     11121     11131     11141     11151     11161     11171     11181     11191     ]
[          |         |         |         |         |         |         |         |         |         |         ]
Dd636      TTTTTATATCTTGTTTTTTGACTTTTTTTGTTTCAGAGGAAAGGTTTTTAGGTTGAAAAGGAGGAAGTTCTATAGAATGAAAACATTCTTCTCCTCCTGAGT
Dd432      ..........................................A.......................................................
Lp_KC875348 ..........................................A.......................................................
Lp_KC875349 ..........................................A.......................................................
Lp_FR821799 ..........................................A.......................................................
Changes NS ..........................................&.......................................................

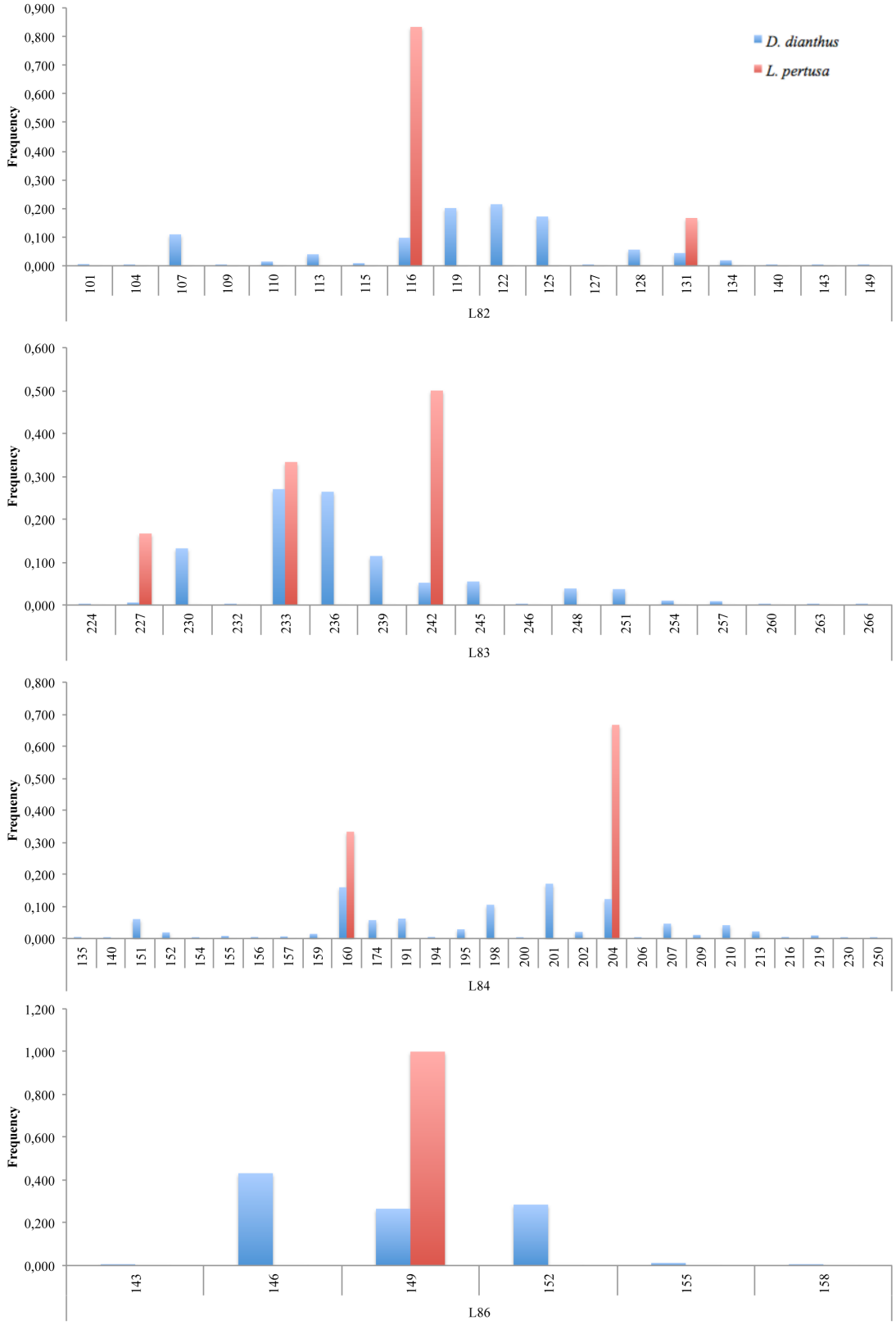
[          11201     11211     11221     11231     11241     ]
[          |         |         |         |         |         ]
Dd636      TTCACACTTATAACGWATTGCCTTTTGTGTTTAGAGTTCTAAGA
Dd432      ..........A.....................................
Lp_KC875348 ..........A.....................................
Lp_KC875349 ..........A.....................................
Lp_FR821799 ..........A.....................T...............
Changes NS ..........S.....................S...............
```

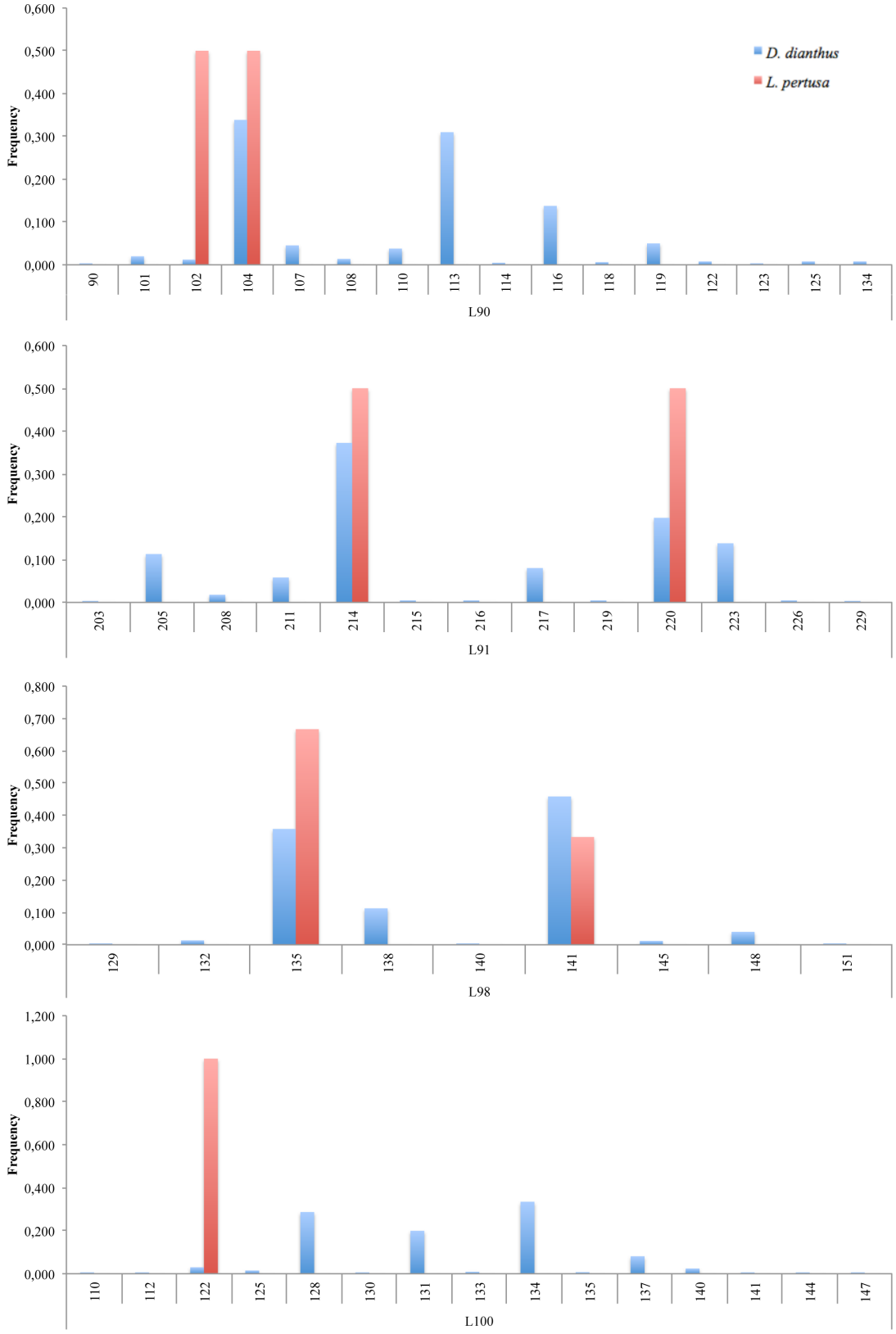
Annexe 4. Allele frequency of *Desmophyllum dianthus* and *Lophelia pertusa* throughout all 30 microsatellites.

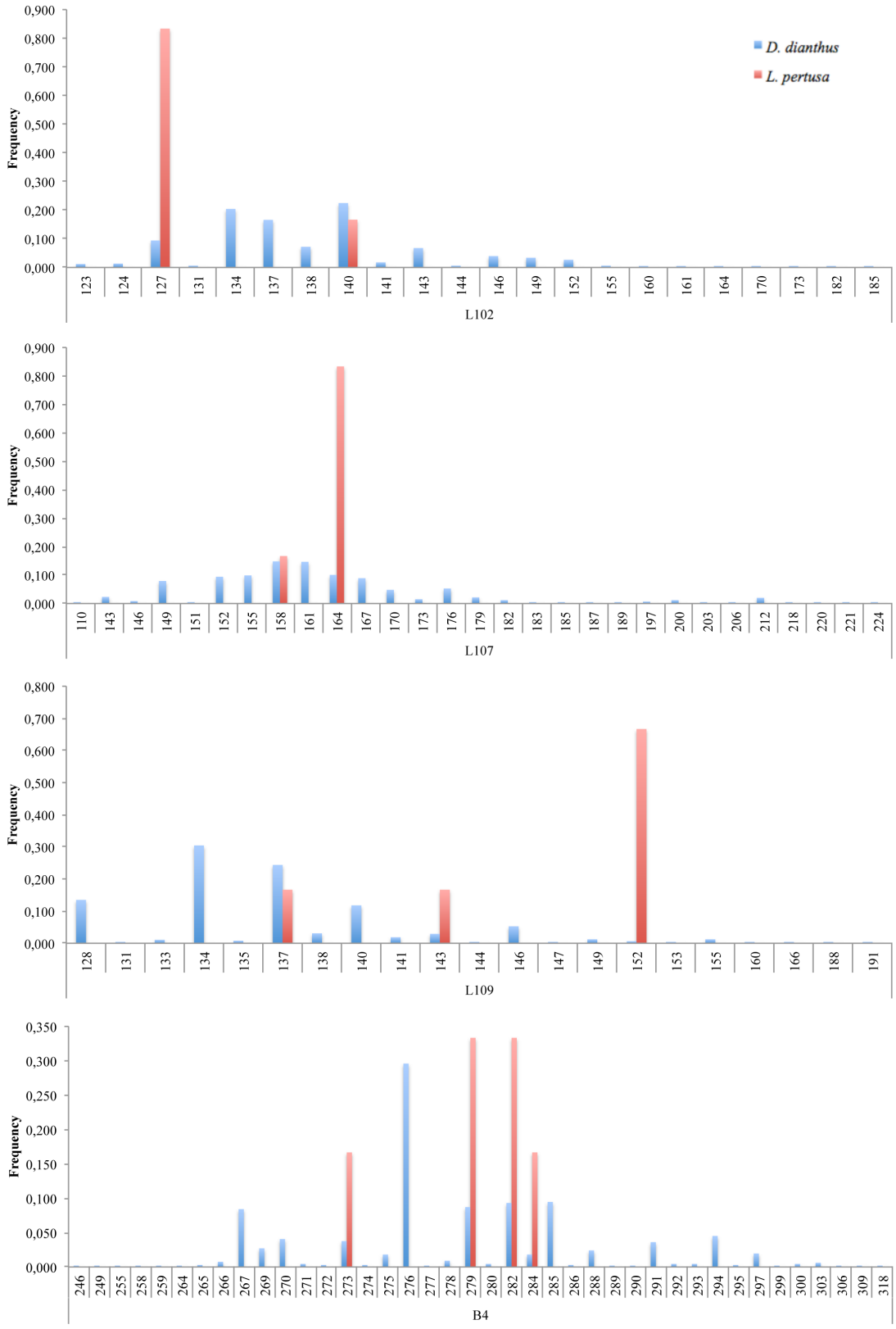

D. dianthus
L. pertusa
Frequency
L7
L13
L16
L22

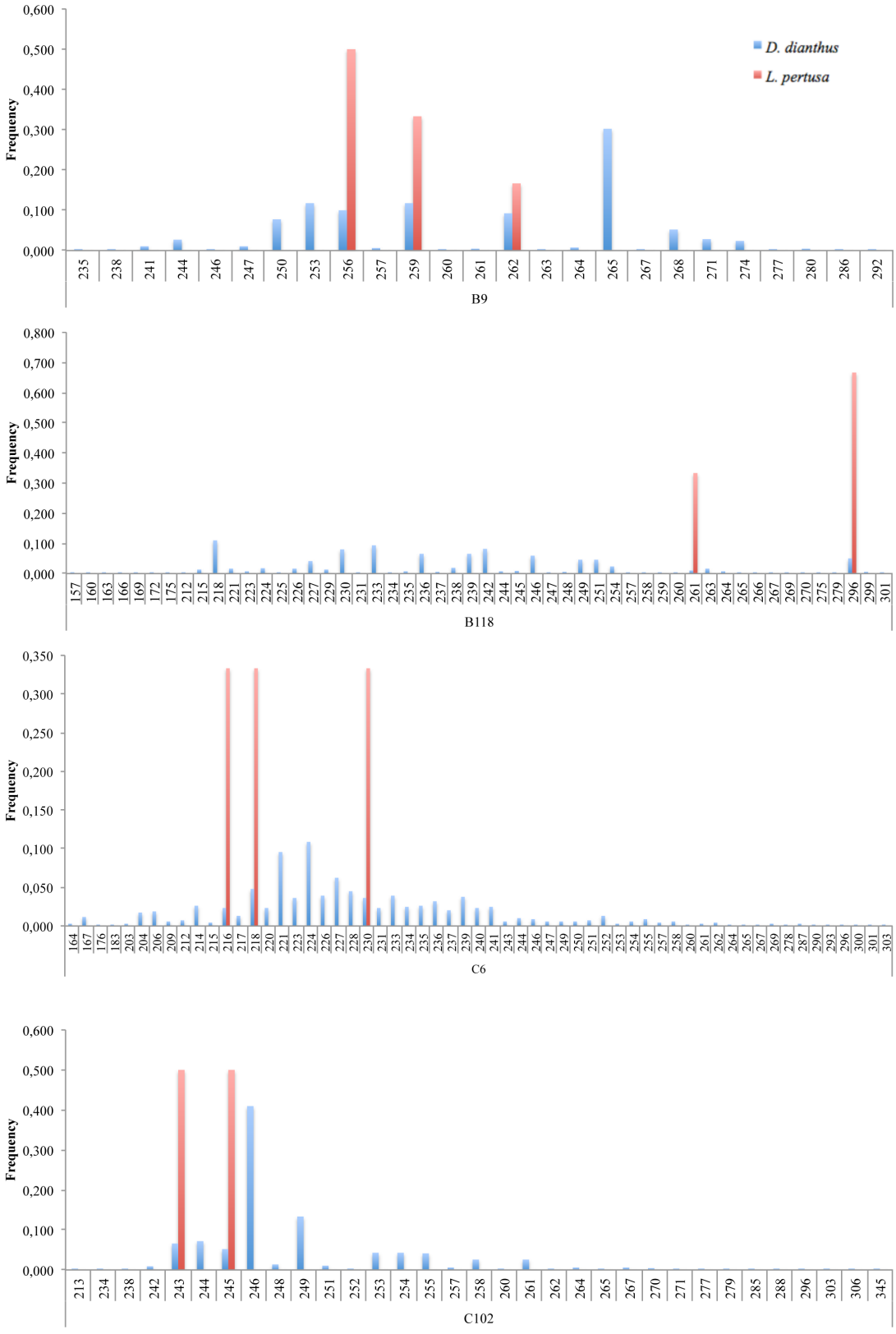

D. dianthus
L. pertusa
Frequency
0,800
0,700
0,600
0,500
0,400
0,300
0,200
0,100
0,000
149 162 165 166 169 170 174 176 178 182 184 186 187 188 190 192 194 196 198 200 201 202 203 206 207 208 210 212 214 216 218 220
L24
1,200
1,000
0,800
0,600
0,400
0,200
0,000
159 164 165 167 168 169 170 171 172 176 177 178 179 180 181 182 183 184 185 187 188 189 190 191 192 193 194 195 196 197 198 199 200 202 203 204 205 206 207 208 210 211 212 213 214 216 217 219 221 223 225 226 227 228 234 240
L34
0,350
0,300
0,250
0,200
0,150
0,100
0,050
0,000
132 135 138 139 142 144 147 150 153 156 159 162 165 168 171 174 177 180 183 186 189 192 195 207 210
L41
0,600
0,500
0,400
0,300
0,200
0,100
0,000
189 191 194 197 199 200 202 203 205 206 208 209 211 212 214 215 217 218 220 221 223 224 227 230 233 236 239 242 245 248 251 254 257 260 263 266 269 273 277 279 284 290
L50

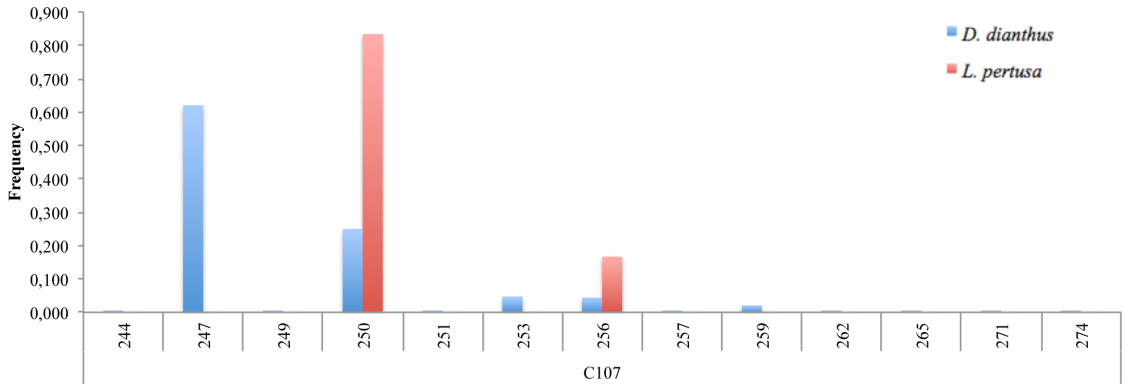

D. dianthus
L. pertusa
Frequency
1,200
1,000
0,800
0,600
0,400
0,200
0,000
115 116 120 124 127 128 130 132 133 136 140 144 148 152
L56
0,800
0,700
0,600
0,500
0,400
0,300
0,200
0,100
0,000
109 113 114 115 117 118 119 121 122 125 129 133 137 139 141 149 153 161
L57
105 109 110 113 117 121 125 129 133 137 141 146 147 152 153 157 163 165 175 178
L58
154 156 158 161 162 163 166 168 169 170 174 176 178 180 182 186 190
L73


D. dianthus
L. pertusa
Frequency
0,900
0,800
0,700
0,600
0,500
0,400
0,300
0,200
0,100
0,000
101 104 107 109 110 113 115 116 119 122 125 127 128 131 134 140 143 149
L82
0,600
0,500
0,400
0,300
0,200
0,100
0,000
224 227 230 232 233 236 239 242 245 246 248 251 254 257 260 263 266
L83
0,800
0,700
0,600
0,500
0,400
0,300
0,200
0,100
0,000
135 140 151 152 154 155 156 157 159 160 174 191 194 195 198 200 201 202 204 206 207 209 210 213 216 219 230 250
L84
1,200
1,000
0,800
0,600
0,400
0,200
0,000
143 146 149 152 155 158
L86


D. dianthus
L. pertusa
Frequency
0,600
0,500
0,400
0,300
0,200
0,100
0,000
90 101 102 104 107 108 110 113 114 116 118 119 122 123 125 134
L90
203 205 208 211 214 215 216 217 219 220 223 226 229
L91
0,800
0,700
129 132 135 138 140 141 145 148 151
L98
1,200
1,000
110 112 122 125 128 130 131 133 134 135 137 140 141 144 147
L100


D. dianthus
L. pertusa
Frequency
0,900
0,800
0,700
0,600
0,500
0,400
0,300
0,200
0,100
0,000
123 124 127 131 134 137 138 140 141 143 144 146 149 152 155 160 161 164 170 173 182 185
L102
110 143 146 149 151 152 155 158 161 164 167 170 173 176 179 182 183 185 187 189 197 200 203 206 212 218 220 221 224
L107
0,800
0,700
0,600
0,500
0,400
0,300
0,200
0,100
0,000
128 131 133 134 135 137 138 140 141 143 144 146 147 149 152 153 155 160 166 188 191
L109
0,350
0,300
0,250
0,200
0,150
0,100
0,050
0,000
246 249 255 258 259 264 265 266 267 269 270 271 272 273 274 275 276 277 278 279 280 282 284 285 286 288 289 290 291 292 293 294 295 297 299 300 303 306 309 318
B4


D. dianthus
L. pertusa
Frequency
B9
B118
C6
C102


0,900
0,800
0,700
0,600
0,500
0,400
0,300
0,200
0,100
0,000
Frequency
D. dianthus
L. pertusa
244
247
249
250
251
253
256
257
259
262
265
271
274
C107